Structure Determination of a Yeast Ribosomal Protein L30 and Pre-mRNA Binding Site Complex by NMR Spectroscopy

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Hongyuan Mao

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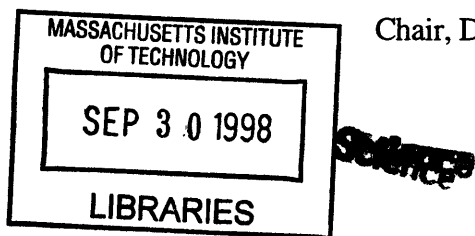MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Signature of Author...

Department of Chemistry

July 25, 1998

Certified by..

James R. Williamson

Professor of Chemistry

Thesis Supervisor

Accepted by..

Dietmar Seyferth

Chair, Departmental Committee on Graduate Students

This doctoral thesis has been examined by a committee of the Department of Chemistry as follows:

Professor JoAnne Stubbe..............   ....

                                                                                                                    Chair

Professor James R. Williamson...........   ....

                                                                                              Thesis Supervisor

Professor Bruce Tidor..

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ABSTRACT

The yeast (*Saccharomyces cerevisiae*) ribosomal protein L30 and its auto-regulatory pre-mRNA binding site provide one of the best examples the critical role of protein-RNA interactions in regulation of RNA processing and control of gene translation. A model system for this interaction, which includes the ribosomal L30 protein and the phylogenetically conserved RNA segment for auto-regulation, was studied using nuclear magnetic resonance (NMR) spectroscopy. The L30 protein recognizes and binds tightly to the stem-internal loop-stem RNA, the recognition elements of which lie mostly on the conserved two-plus-five asymmetric purine-rich internal loop. NMR characterizations were carried out on both the free and bound forms of the protein and the RNA. Detailed analyses of the protein revealed that the main architecture, a four-stranded β-sheet sandwiched between four α-helices, is present both in the free and in the bound form. There are however, substantial local perturbations that accompany RNA binding, the largest of which have been mapped onto the loops connecting Strand A and Helix 2, Strand B and Helix 3, Helix 4 and Strand D. In contrast to the protein, the internal loop of the RNA undergoes significant changes upon complex formation, and the most distinct observation was the formation of the G11·G56 reverse Hoogsteen mismatch pair. Structure modeling using simulated annealing in restrained molecular dynamics was carried out in X-PLOR. Detailed analyses of the complex structure reveal that the protein recognizes the RNA mostly along one side of the internal loop with five purines. The interactions are divided further into two sections. One region consists of mostly aromatic stacking and hydrophobic contacts from Leu25, Phe85 and Val87 of the protein to G56 of the RNA. The other region consists of mostly specific contacts, which include recognition of A57 by Asn 48, and G58 by Arg 52. The L30 protein-RNA complex structure thus determined using NMR spectroscopy not only provides a detailed insight for understanding the structure-function relationship regarding the yeast auto-regulation, it also further demonstrates the important role of the protein-RNA interaction in controlling RNA processing and gene translation.

Thesis Supervisor: James R. Williamson

Title: Professor of Chemistry

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As I was growing up, my Dad, an old-fashioned organic chemist, used to tease me of not being able to picture the three-dimensional structures of his organic compounds. I would never have thought of becoming a structural chemist until seven years ago, when I met Dr. Allen Bedford, at the time a graduate student at Temple University. His fascination with hemoglobin, a well-studied small protein, could always stir up some serious debates about salt bridges, hydrogen bonding, and hydrophobic interactions, *etc.*, and I wished that I had known more about the structures and functions of macromolecules. It wasn't a tough choice then, when I came to MIT and joined Dr. James R. Williamson's Laboratory in order to fulfill my curiosities. Looking back, I have to say I feel quite fortunate to be involved in his lab, since Jamie's ambition and fascination with science always pushed me for tough challenge throughout my graduate career.

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I would like to especially thank Dr. Jeffrey Orr, who has provided me with enormous love and support over the years. Your patience and carefulness has helped me through many of the writing crises. I am so relieved that we have come through the "danger zone" writing the thesis.

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Table of Contents

CHAPTER 1. Introduction

1.1. Protein-RNA Interactions

1.1.1. Importance of protein-RNA interactions

The central dogma in Biology indicates that genetic information is passed from DNA to RNA through transcription and from RNA to protein through translation (Crick, 1958). Specific protein-RNA interactions participate in many of these processing events and play crucial roles in gene regulation through transcription, RNA processing, transport and translation. Our understanding of how sequence and structure unite to form determinants required for protein-RNA recognition has dramatically increased recently through many areas of investigation, including molecular biology, biochemistry, macromolecular crystallography and high resolution nuclear magnetic resonance spectroscopy (NMR). Protein-RNA interactions are essential in many processes, including the areas discussed below that have been subjected to extensively studied in recent years.

1.1.1.1. Transcription control

There are several well-established examples that show transcription is either positively or negatively regulated by protein-RNA interactions. The human immune-deficiency virus (HIV) regulatory protein Tat binds to the trans-activation region (TAR) in the 5'-long terminal repeat (LTR) of a growing viral messenger RNA and promotes RNA polymerase transcription efficiency (Berkhout *et al.*, 1989). The binding of the bacteriophage N protein to a small RNA element in nascent RNA transcript and subsequent interaction with other cellular proteins produce a termination resistant complex (Konrad, 1970). The biosynthesis of tryptophan (trp) in *Bacillus subtilis* is controlled by an attenuation mechanism, whereby the attenuation protein (TRAP) binds to the 5'-upstream translation region (UTR) of mRNA, and allows the

downstream transcript to form a hairpin structure that leads to transcription termination in responds to the high level of tryptophan (Witherell *et al.*, 1991).

1.1.1.2. RNA processing

Most RNAs synthesized in eukaryotic cells undergo extensive processing (i.e. capping, splicing, polyadenylation and post-transcriptional modification) before they reach the cytoplasm for protein synthesis. While these premessenger RNAs (pre-mRNAs) are nascent and remain attached to their DNA templates, they quickly become associated with certain RNA binding proteins. This ubiquitous protein-bound state is referred to as heterogeneous nuclear ribonucleoprotein (hnRNP). It is in this form that pre-mRNAs undergo splicing and other processing reactions that convert them into mature mRNA molecules. The best known example is the spliceosome composed of a number of small nuclear RNAs (snRNAs) and many small nuclear ribonucleoproteins (snRNPs) that are involved in diverse aspects of nuclear RNA processing, including specific intron recognition and removal from messenger RNA precursors (pre-mRNAs).

1.1.1.3. RNA transport

All mRNAs synthesized in the nucleolus are exported through nuclear-pore complexes into the cytoplasm for protein synthesis. While it is unclear whether most RNAs are exported through their association with hnRNP proteins, there is substantial evidence to indicate that the Rev protein of HIV can interact with the Rev responsive element (RRE) RNA to inhibit splicing and facilitate the partially spliced and/or unspliced viral mRNA export (Felber *et al.*, 1989).

1.1.1.4. Ribosome assembly

About 85 percent of cellular RNAs are found in ribosomes, and the absolute amount is greatly increased in cells engaged in large-scale protein synthesis. There are extensive sequence specific protein-RNA contacts that govern the hierarchical process of its assembly. Precise

incorporation of more than 50 proteins into its rRNA framework is absolutely required for the ribosome to function in protein synthesis.

1.1.1.5. Protein synthesis

In the cytoplasm, protein-RNA interactions play a central role in protein synthesis. The fidelity of translation depends on specific aminoacylation of fifty different transfer RNAs (tRNAs) by different aminoacyl-tRNA synthetases (aaRS). The actual decoding of the genetic information including initiation, elongation and termination is performed by the ribosome, which itself is a large protein-RNA complex. Other protein-RNA interactions also participate in regulation of protein synthesis. For instance, most prokaryote ribosomal proteins use a translational feedback inhibition mechanism, whereby excess ribosomal protein binds to the 5' region of its polycistronic mRNA and inhibits its production. Another example is the MS2 bacteriophage coat protein, which not only performs sequence-specific RNA encapsidation but also represses the translation of replicase by binding a hairpin structure in the Shine-Dalgarno sequence of the viral replicase mRNA. This process plays an important role as a biological switch from the replication phase to the assembly phase of the phage life cycle.

The above protein-RNA complexes are present in cells in very low quantities, however, obtaining large quantities of many RNA-binding proteins and cognate RNAs, which are necessary for many structural studies, has been made possible through molecular cloning and overexpression of these molecules in bacteria. Furthermore, large quantities of RNA can be made through *in vitro* T7 RNA polymerase enzymatic synthesis, directed by templates derived from plasmids purified from bacteria or oligos through chemical synthesis. These technical advances have made it possible to prepare homogenous samples in a reasonable time period thereby facilitating the structural studies using X-rays crystallography and nuclear magnetic resonance (NMR) spectroscopy.

1.1.2. Typical architectures for RNA binding protein

High-resolution structures of various RNA binding proteins have revealed unexpected sequences yet show structural relationships among these proteins. Although the specificity of a protein for a particular RNA depends on the specific function, functional roles are made possible by presenting the functional groups in properly folded protein structures. Comparative structural analysis of proteins from the protein synthesis (i.e. the aminoacyl-tRNA synthetases, ribosomal proteins and elongation factors), spliceosomal, RNA viral and other RNA-binding systems have categorization of binding domains into a few discrete motif. Among them, the β-sheet is a common structural element in many cases, while pure α-helical proteins are much less frequently observed. In particular, most RNA binding-domain belongs to an α/β family of proteins. These homologous structures contain α/β motif consisting of a three-stranded anti-parallel β-sheet and an α helix. This motif is found in the most commonly observed RNA-binding domains, which include the ribonucleoprotein (RNP) domain, the double stranded RNA binding domain (dsRBD), the K homology (KH) domain and the S1 domain. For example, the RNP domain has been found in over 200 proteins involved in RNA processing, transport and metabolism (Mattaj, 1993; Nagai *et al.*, 1995). It consists of a four-stranded antiparallel β sheet flanked on one face by two α helices. The RNP1 and RNP2 motifs are located in the two middle β strands and play a crucial role in RNA binding. The dsRBDs have an α-β-β-β-α topology and fold into a three-stranded anti-parallel β sheet packed on one side by two α helices. Both loop 2 and loop 4 are conserved in this family and are implicated in RNA binding. The K homology (KH) domain (Nagai, 1996) found in the heterogeneous nuclear RNP (hnRNP) K has also been identified in a number of proteins associating with RNA. A NMR study on the structure of the KH domain of human vigilin, a protein thought to be involved in tRNA transport, has shown that it contains a three stranded anti-parallel β-sheet and three helices. The fourth RNA-binding motif known as the S1 domain (Bycroft *et al.*, 1997) was first identified in *E. coli* ribosomal protein S1 and is also present in RNase E, RNase II, NusA and some other RNA-associated proteins. The S1 domain consists of a five-stranded anti-parallel β-sheet forming a β-barrel. It appears that many RNA-binding proteins use β-sheets as binding surface for hairpins or single-stranded RNAs. Aromatic side chains on the β-sheets provide a platform for the RNA bases. The anticodon-

binding domains of GlnRS, U1 snRNP protein A and the coat protein of the phage MS2 exemplify this structural arrangement. U1A, in one of them, it is well established that the aromatic residues participate in stacking interactions with RNA bases (Liljas & al-Karadaghi, 1997). In all these cases the RNA is unraveled after a conformational change induced by the protein such that the bases are splayed out to maximize specific and other interactions with the protein. The new conformations are stabilized by inter- and intramolecular hydrogen bonding. In addition, loop regions of the protein play important roles in specific recognition of RNA bases.

1.1.3. RNA structural elements

Proteins can recognize and interact with several feature of RNA structures. The overall shape and fold of an RNA molecule, or a segment of specific nucleotide sequence elements are all important in interacting with protein. While double stranded helical regions are commonly observed in RNA structure, regular A-form helices less frequently serve as recognition elements in a protein-RNA complex. Compare to the B-form DNA, which has a wide and shallow major groove facilitating sequence specific protein recognition, the A-form RNA duplex contains a deep and narrow major groove that is largely inaccessible (Seeman *et al.*, 1976). Hence, RNA almost never exists as a long uninterrupted double helix. However, if the major groove of RNA is sufficiently distorted such that the major groove is widened, it can accommodate protein recognition elements, as exemplified by the Rev-RRE complex (Battiste *et al.*, 1996). In general, protein helices interact preferentially with the minor groove of RNA, in contrast to the case for DNA-binding proteins, while protein loops and β-strand elements have been observed to interact with the major groove of RNA.

Despite of its limited functional group diversity, base pairs in RNA expand the standard palette from the canonical Watson-Crick base pairs to noncanonical two hydrogen bond pairs (*i.e.* Hoogsteen, homopurine, homopyrimidine, and wobble base pairs, *et al.*). They further extend to include one-hydrogen bond base pairs, water-mediated base pairs, protonated base

pairs, as well as base-2'OH and base-phosphate hydrogen bonding interactions. These nonstandard base pairs are quite common and often phylogenetically conserved, and provide RNA recognition sites at both major and minor grooves. Furthermore, the array of RNA secondary structures is also increased from standard double helices to include elements such as hairpin, asymmetric internal bulge, two stacked helices, three-way junction and four-way junction (Figure 1.1.1). These secondary structural elements not only serve as building blocks for RNA tertiary structure formation; they also widely act as sites for protein recognition. Bulges or internal loops inserted into a helix render a widened major groove, thus facilitating tertiary contact and protein recognition. While limited amounts of structures of RNAs in complex with protein are currently available, the functional elements of these RNA structures have been studied extensively. RNA motifs that have already been observed in multiple RNA structures include: A platforms (Cate & Doudna, 1996), base zippers (Correll *et al.*, 1997), interstrand purine stacking (Pley *et al.*, 1994), reversed sugars (Szewczak *et al.*, 1993) and U turns, including GNRA tetraloops (Heus & Pardi, 1991b), UUCG tetraloops (Varani *et al.*, 1991) and pseudoknots (Kolk *et al.*, 1998).

1.1.3.1. RNA hairpins

RNA hairpins are a common feature in RNA, particular in rRNA. Approximately 70% of the 16S rRNA folds into 31 different stem loops (Varani, 1995). A hairpin loop is formed when a single strand flips back on itself to form a double-stranded region leaving the bases at the turn unpaired. Exceptionally stable hairpins with compact shapes and specific hydrogen bonding patterns usually serve as nucleation sites and tertiary contacts for RNA folding (*e.g.*, GNRA, UUCG, and CUUG tetraloops; Varani, 1995; Cate & Doudna, 1996). Many hairpin loops that can undergo conformational changes upon binding play very important roles in protein recognition and gene regulation. The seven-member anti-codon loop of tRNA[Phe] is recognized by the phenylalanyl-tRNA synthetase during aminoacylation (Saenger, 1984). The HIV-1 trans-activating region (TAR) RNA is a bulge-stem-loop hairpin that activates transcription upon Tat binding (Frankel, 1992). The hairpins are also one of the control elements for autoregulation. T4 DNA polymerase can block its synthesis by binding to a hairpin upstream of the Shine-
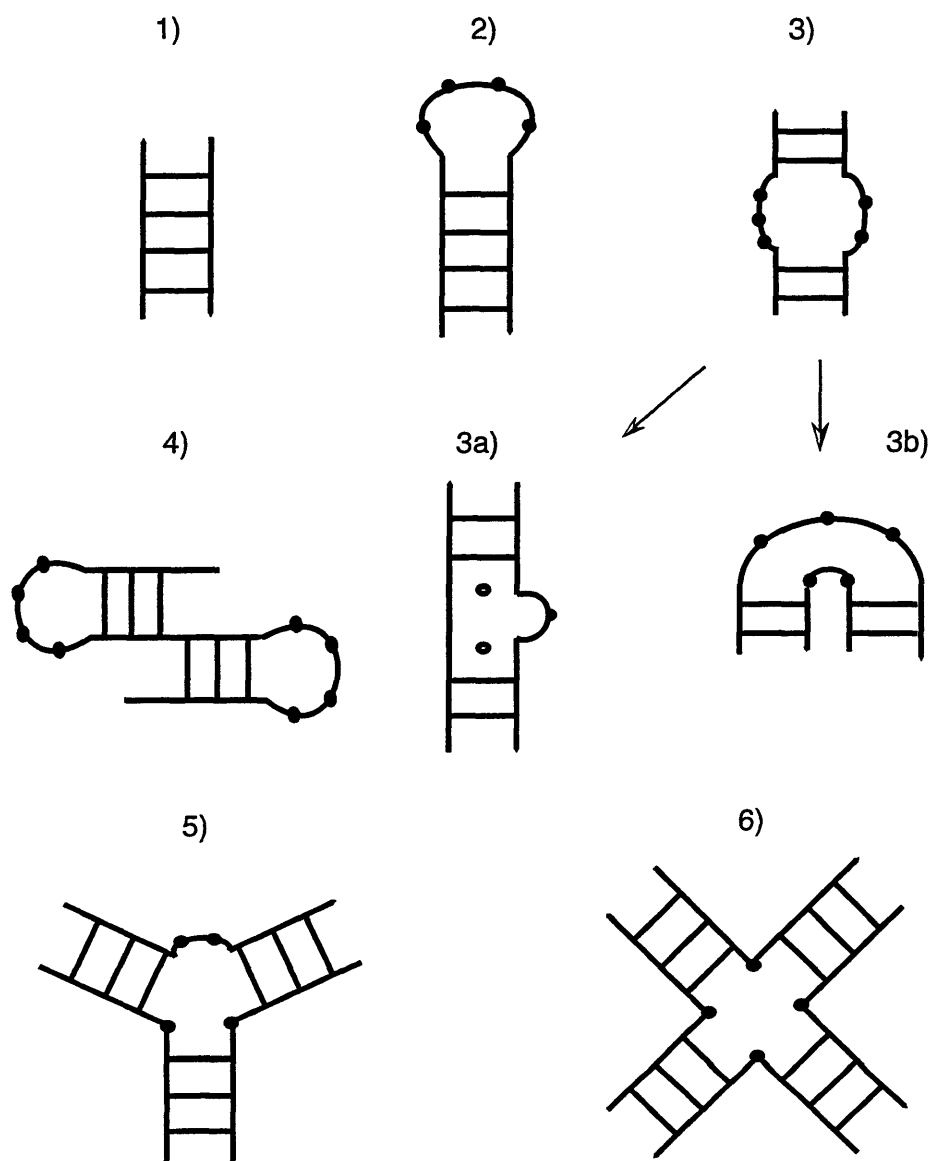
Figure 1.1.1: Typical secondary structural elements in RNA: 1) helix, 2) hairpin, 3) internal loop, including 3a) wobble base pair with bulge loop and 3b) bent helix, 4) pseudoknot, 5) three-way junction, and 6) four-way junction.

Dalgarno sequence of mRNA. Perhaps the most well known case is the iron responsive element (IRE), where a stem-hairpin loop structure interacts with different proteins to increase ferritin synthesis and decrease transferrin receptor synthesis as the iron concentration increases. Hairpin RNAs are also important in all steps of translation, but primarily in controlling the termination. For examples, in prokaryotes, a termination signal is comprised of an RNA hairpin followed by six to eight uridines, whereas in lambda bacteriophages (λ, P22 and ϕ21), a small hairpin (box B) in *nut* site RNA is important for anti-termination regulation (Greenblatt *et al.*, 1993).

1.1.3.2. Internal loops and bulges

Internal loops and bulges are sequence interruptions in a double-strand helix where standard Watson-Crick base pairs cannot form. As a result, there is a potential for accessibility of the otherwise deep and narrow RNA major groove for protein recognition. Many important biological functions of RNA are associated with internal loops and bulges. Secondary structures based on Watson-Crick base pairing often give people the false impression that these internal loops are merely floppy regions flanked by helical stems. However, structural studies have shown that the loop regions are often highly structured through formation of mismatches. G-A mismatches are commonly found in rRNAs, usually in the thermodynamically stable imino hydrogen-bonded conformation and sheared conformation forms (Gautheret *et al.*, 1994). An imino hydrogen-bonded G-A pair and a *trans*-conformation G-G mismatch in RRE internal loop open up the major groove that accommodate an α-helical Rev peptide (Battiste *et al.*, 1996).

1.1.3.3. Pseudoknots

Pseudoknots are a popular class of RNA structure, defined as a set of base pairings that cross an existing secondary structure helix. Pseudoknots offer a number of sites for interaction with protein, with the most intriguing function in translational frameshifting demonstrated in mouse mammary tumor virus (MMTV) (Chamorro *et al.*, 1992) and infectious bronchitis virus (IBV) (Brierley *et al.*, 1991). The pseudoknot structure of turnip yellow mosaic virus (TYMV) (Kolk *et al.*, 1998) shows the intimate interaction of two loops to the helical region while

spanning across major and minor groove of the RNA. The structure resulting from this interaction between the minor groove and single-stranded RNA at helical junctions displays internal mobility, which may be a general feature of RNA pseudoknots that regulate their interaction with proteins or other RNA molecules.

1.2. The Ribosome as a Rich Source of Protein-RNA Interaction

1.2.1. Complexity of the ribosome

All ribosomes consist of small and large subunits. Each subunit is a complex between one or more large ribosomal RNA (rRNA) molecules and a number of relatively small, predominately basic proteins. Prokaryotic ribosomes (*e.g. E. coli*) consist of 30S and 50S subunits, comprised of a 16S rRNA and 21 proteins, and 5S and 23S rRNAs and 32 proteins, respectively (Wittmann, 1982). Eukaryotic (*e.g.* yeast) ribosomes are larger and are made of 40S and 60S subunits, which comprise an 18S rRNA and about 32 proteins, and 5S, 5.8S and 28S rRNAs and about 46 proteins, respectively (Kozak, 1983).

The ribosome is a large RNA-protein complex where the process of protein synthesis takes place, including initiation, elongation and termination. A detailed knowledge of the ribosome structure will be required before its function and mechanisms can be fully understood. The organization and low resolution structure of the prokaryotic ribosome and its subunits have been studied using *in vitro* assembly, electron microscopy, neutron scattering and diffraction, and chemical probing (Lake, 1985; Moore, 1992). Crystallography and NMR methods have provided tremendous insight into the detailed features of several ribosomal proteins and fragments of rRNA. There are more than a dozen partial or full structures out of more than 50 proteins from prokaryotic ribosomes available: S1, S5, S6, S7, S8, S15, L1, L6, L9, S17, L14, L22, L30, the L7/L12 C-terminal domain, L11 C-terminal RNA recognition domain (see reviews in (Liljas & al-Karadaghi, 1997; Arnez & Cavrelli, 1997)). A number of common structural

features are seen in these ribosomal proteins. The most frequently occurring motifs are based on anti-parallel β-sheets. One recurring motif is the split β-α-β, or RNA recognition motif (RRM), first found in the RNA binding domain of the protein U1A and in many other proteins, including ribosomal proteins S6, L1, L6, L7/L12, L9, L22 and L30. Another type of fold, based on an anti-parallel β-barrel, is found in S17 and as the repeated domain in the largest ribosomal protein S1. Purely helical proteins seem to be in a clear minority, with S15 alone known to belong to this class. The three-dimensional structures reveal that the distribution of positive charge (due to lysine and arginine residues) over the surface of the proteins is far from even, rather conserved patches of positive surface potential are observed.

1.2.2. Regulation of ribosome biosynthesis

The biosynthesis of ribosomes requires the coordination of a large number of steps which must be carefully orchestrated. These include transcription of ribosomal RNAs (rRNA) and messenger RNAs (mRNA) encoding ribosomal proteins, translation of these mRNAs into more than 50 ribosomal proteins in cytoplasm, translocation of proteins into nucleolus, assembly of ribosomal proteins with rRNAs in the nucleolus, and export of the completed ribosomal subunit to the cytoplasm. In *E. coli*, the ribosomal protein genes are clustered in large operons, and the transcription of most ribosomal protein genes is constitutive by the sole species of RNA polymerase. The regulation of synthesis of ribosomal proteins commonly occurs at the level of translation whereby excess ribosomal protein binds to its polycistronic mRNA to inhibit translation of its several encoded proteins (Nomura *et al.*, 1984; Jinks-Robertson & Nomura, 1987). In contrast, eukaryotic cells (e.g. yeast) have three classes of RNA polymerases: RNA polymerase I makes a 35S precursor ribosomal RNA; polymerase II makes mostly mRNA; and polymerase III makes transfer RNA (tRNA), 5S rRNA and a few other small RNA molecules (Watson *et al.*, 1987). In yeast, regulation of ribosome synthesis occurs at different steps. First, since all mRNAs are monocistronic and are scattered throughout the genome, the promoter of all the ribosomal protein genes have coevolved to provide roughly equimolar amounts of mRNA (Kim & Warner, 1983). Furthermore, many of the ribosomal protein genes in yeast cells contain

a single intron that is uncommon for other yeast proteins. The production of the ribosomal

proteins can be auto-regulated at both premessenger RNA (pre-mRNA) splicing and mRNA

translation levels (Warner, 1989), this ultimately controlling the balanced production of the

ribosome.

1.2.3. The yeast ribosomal protein L30 and its autoregulation RNA binding site

The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) ribosomal protein L30[1] (formerly

know as L32) and its autoregulation RNA binding site is one of the best examples that

demonstrates the critical role of protein-RNA interactions in regulation of RNA processing and

control of gene translation. Despite the fact that the functional role of L30 protein in the yeast

60S ribosomal subunit is unclear at the moment, its autoregulation that influences splicing of pre-

mRNA, translation of its mRNA, and the processing of rRNAs have been best characterized

through many *in vivo* studies.

1.2.3.1. The L30 protein

The L30 protein in S. *cerevisiae* is a small (104 amino acids) but essential protein in the

60S ribosomal subunit (Daveba & Warner, 1987). Homologs have been found in organisms

from eukaryo and archaea but not prokarya, of which the protein sequence alignment is shown in

Figure 1.2.1 using BLAST program (Altschul & Lipman, 1990). Less information is available

for the exact rRNA binding site of the L30, because of the more complex eukaryotic ribosome

components and assembly pathway. Nevertheless, L30 appears to interact with at least three

distinct RNA molecules to influence different elements of RNA processing and function. It

autoregulates the splicing of L30 pre-mRNA, translation of its mRNA, and influences the

processing of the 35S pre-rRNA for 60S ribosomal subunits. Among these functions, the

feedback inhibition of pre-mRNA splicing is the most-studied and best understood system, which

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[1] The yeast ribosomal protein L30 was originally called L32, however, it has be renamed by the new nomenclature
for the yeast ribosomal proteins introduced by several laboratory (Mager *et al.*, 1997).

has been identified as the association of a stem-loop-stem RNA with the L30 protein, and interference with formation of a complete spliceosome. The RNA has been mapped out through phylogenetic comparisons between two yeast species, namely *S. cerevisiae* and *Kluveromyces lactis* (*K. lactis*), and extensive mutational analysis (Eng & Warner, 1991), encompassing the 5'end of the exon, the first AUG codon, and the 5' end of the intron (Figure 1.2.2). A similar RNA structure with only a single nucleotide variation can also form within the spliced mRNA, thus providing the transnational regulation (Li *et al.*, 1996). It is very likely that the L30 protein interacts with these RNAs by similar mechanisms, but how it binds to the RNA is still unclear. Furthermore, the most important functional role of the L30 protein is located within the ribosome. Although the exact binding site is not yet known, it is likely that the binding is similar to the mechanism used with the auto-regulation site. This has precedence form other ribosomal proteins (Nomura *et al.*, 1980).

1.2.3.2. The L30 protein pre-mRNA binding site

Extensive *in vitro* and *in vivo* biochemical analyses have provided valuable information on the secondary structure content of the pre-mRNA substrate of the L30 protein. Studies including mutational analysis, chemical and enzymatic probing, and modification interference experiments support the notion that a purine-rich asymmetric internal loop between two helical stems is important for protein recognition (White & Li, 1995) (Figure 1.2.3, panel **a**). Further experiments utilizing SELEX (Systematic Evolution of Ligands by EXponential enrichment) show that a two-plus-five (two residues on one side, five on the other) internal loop motif with conserved 5'-GA-3' dinucleotides on both sides of the internal loop is critical for protein binding. Six out of the seven nucleotide positions show a preference for purines (Li & White, 1997) (Figure 1.2.3, panel **b**).

1.2.3.3. Biochemistry of the pre-mRNA binding site

While *in vitro* mutagenesis studies and phylogenetic comparisons have suggested the existence of two helical regions in the pre-mRNA binding site, *in vitro* chemical and enzymatic

```
                                                                        34
  yeast   ------MAPV KSQESINQKL ALVIKSGKYT LGYKSTVKSL
  klula   ------MAPA KNQENINQKL ALVVKSGKYS LGYKSTVKSL
  schpo   MASVVTKKSK KSGDTINAKL ALTMKSGKYV LGYKSTLKTL
  chick   --MVAAKKTK KSLESINSRL QLVMKSGKYV LGYKQTLKMI
  human   --MVAAKKTK KSLESINSRL QLVMKSGKYV LGYKQTLKMI
  trybb   ----MAKKVK SKVDTINTKI QLVMKSGKYV LGTQQSLKTL
  leima   ----MAKKTK SKVNTINAKL QLVMKSGKYV LGTQQALTTL


                                                                        74
  yeast   RQGKSKLIII AANTPVLRKS ELEYYAMLSK TKVYYFQGGN
  klula   RQGKAKLIII AANTPVLRKS ELEYYAMLSK TKVYYFQGGN
  schpo   RSGKAKLILI AGNCPPLRKS ELEYYAMLSK ANVHHYAGTN
  chick   RQGKAKLVIL ANNCPALRKS EIEYYAMLAK TGVHHYSGNN
  human   RQGKAKLVIL ANNCPALRKS EIEYYAMLAK TGVHHYSGNN
  trybb   RQGKSKLVVI SANCPPIRKA EIEYYCTLSK TPIHHYSGNN
  leima   RQGKSKLVVI ANNCPPIRRA EVEYYCTLSK TPIHHYSGNN


                                                                        105
  yeast   NELGTAVGKL FRVGVVSILE AGDSDILTTL A------
  klula   NELGTAVGKL FRVGVVTILD AGDSDILTAL A------
  schpo   IDLGTACGKL FRVGVLAITD AGDSDILDA- -------
  chick   IELGTACGKY YRVCTLAIID PGDSDIIRSM PEQTSEK
  human   IELGTACGKY YRVCTLAIID PGDSDIIRSM PEQTGEK
  trybb   LDLGTACGRH FRACVLSITD VGDSDITSA- -------
  leima   LDLGTACGKP FRTCVLSVTN VGDSDIAT-- -------
```

Figure 1.2.1: Sequence alignments of 60S ribosomal protein L30 from various species, including **a**, *Saccharomyces cerevisiae*; **b**, *Kluyveromyces lactis*; **c**, *Schizosaccharomyces pombe*; **d**, chicken; **e**, human; **f**, *Trypanosoma brucei brucei*; **h**, *Leishmania major*. The numbers refer to the *S. cerevisiae* sequence. Conserved residues are hightlighted in red, relatively conserved hydrophobic, charge residues are highlighted in blue and green, respectively. Alignments were performed using the program BLAST (Altschul *et al.*, 1990) for protein sequences in the NCBI database.
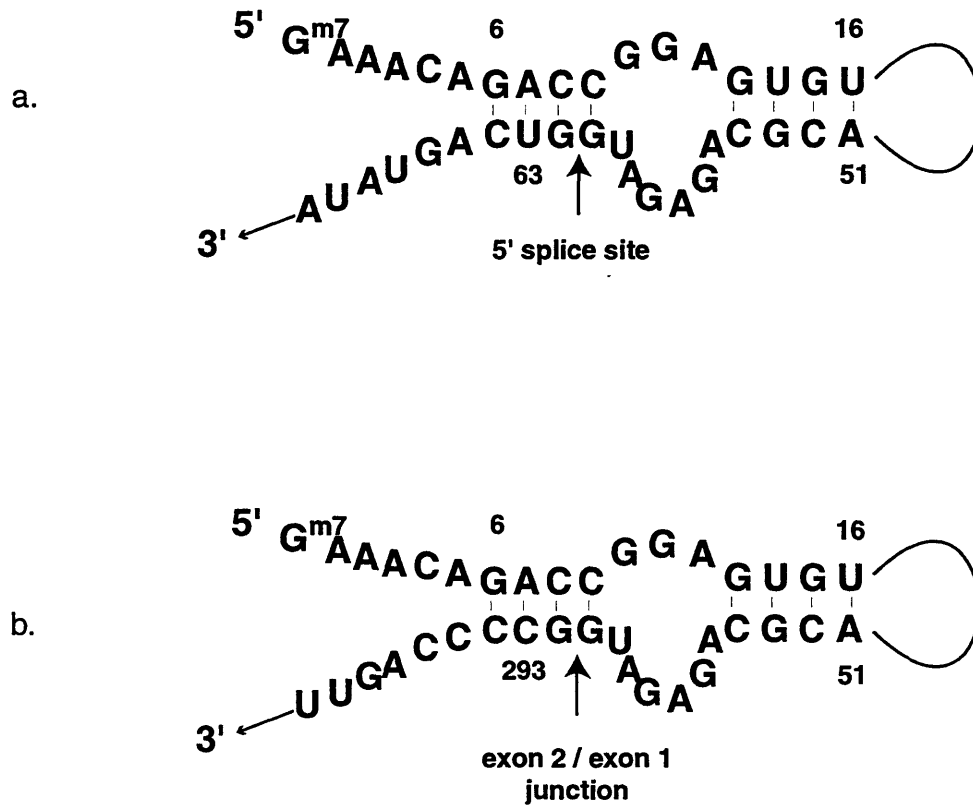
Figure 1.2.2: Proposed RNA secondary structures involved in autoregulation of synthesis by yeast ribosomal protein L30 (Li *et al.*, 1996): a, pre-mRNA binding site for regulation of splicing; b, mRNA binding site for feedback regulation of translation. Note that the U63 in a is replaced by C293 in b, and the unpaired bases at the 3' ends are different. Currently, the rRNA binding site in the 60S subunit of the ribosome is unclear.

a.

```
        U   U
    U       A
  U           U  50
    U – A
 15 G – C
    U – G
   (G)– C  ↗↗
     (A)      (A)(G)
    (G)           A  57
                   (G)
 10  G              (A)
    C – G   U
    C – G
    A – U
    G – C
        A  65
      g
        G
     g        3'
    g
  5'
```

b.

```
        N – N  ⎤
        N – N  ⎥  2-4 base pairs
        N – N  ⎥
        N – N  ⎦  55
    12  A     N   G
    G             C  57
                  G
 10  G          U A
    C – G  ⎤
    N – N  ⎥
    N – N  ⎥  5-7 base pairs
    N – N  ⎥
    N – N  ⎦
   5'    3'
```

MiniL32 RNA   **RNA Aptamer**
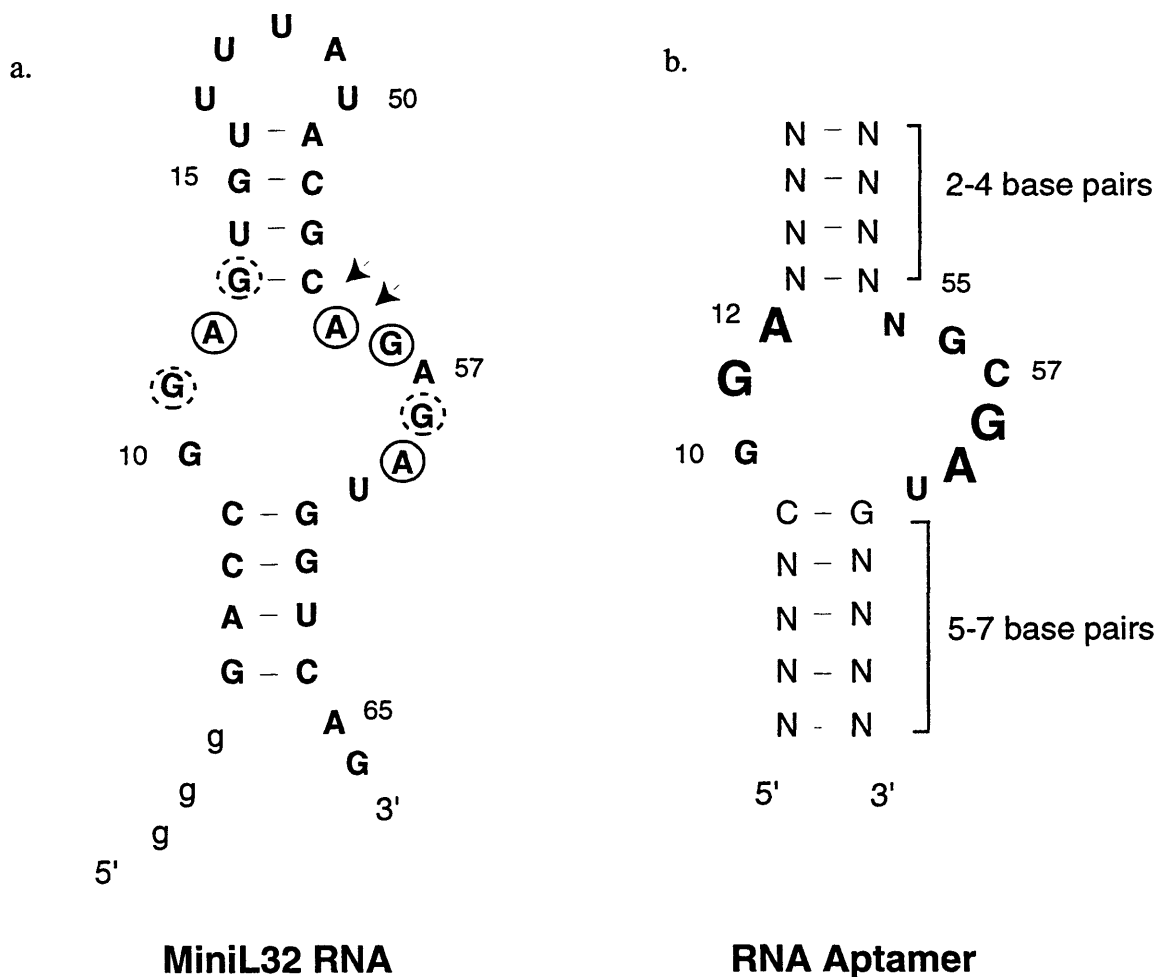
Figure 1.2.3: Schematic representations of results from biochemical studies of L30 protein pre-mRNA binding site. Panel a shows minimal RNA fragment used in biochemical analyses. Circles (open or dashed) and arrows represent the respective chemical modifications of bases and phosphates that are important for L30 protein binding (Li *et al.*, 1995). Open circles represent nucleotides protected from cleavage upon protein binding. Lower case letters represent non-native nucleotides. Panel b shows secondary structure scheme of RNA aptamer summarizing the results of an *in vitro* SELEX analysis of L30 RNA (Li *et al.*, 1997). The numbering of nucleotides in the internal loop region follows the natural L30 transcript as in MiniL32 RNA. N represents any nucleotide, while bases in bold characters represent either the absolutely conserved (larger font), or most frequently observed (smaller font) nucleotides.

probing confirmed the existence of two helical stems, and further identified the internal loop region as the important binding site. These *in vitro* studies were carried out mostly on model systems, including MiniL32 RNA (a small binding element), and other similar constructs (White & Li, 1995). In the absence of protein, G10, G11, A12, A55, A59, and U60 in the purine rich region are all intrahelical and probably paired, but N-7 position of A57 is reactive to diethylpyrocarbonate (DEPC), and the N-7 position of G56 reacts strongly with the nickel macrocycle [(2,12-dimethyl-3,7,11,17-tetraazabicylo[11.3.1]heptadecal(17),2,11,13,15-pentaenato)nickel hexafluorophosphate] (also called NiCR), indicate a possible bulge region. In the presence of protein, G11, A12, G13, A55, G56, G58 and A59 are protected from chemical modification. Carboxethylation of the N7 positions of A12, A55 and A59 in the loop region abolishes protein binding while modification of the N7 positions of G15, A49, A51 in the helical region and A57 in the loop region has no effect on protein binding. Modification of the N7 position of A57 does not interfere with protein binding, but is protected from DEPC attack upon protein binding. RNAs with deletion of either A57 or G56 failed to bind MBP-L30[2], a fusion construct used for studying the L32 protein (White *et al.*, 1995). Modification of the phosphate backbone with ethylnitrosourea (ENU) shows that backbone positions 10 to 12 and 55 to 58 of the internal loop are important for protein binding. Footprinting with Fe(II)-EDTA, which generates hydroxyl radicals and attacks the ribose ring, shows that nucleotides 11 to 13 and 55 to 59 are protected from hydroxyl-radical attack by binding of the fusion protein. Filter binding experiments established that the binding stoichiometry is one RNA per fusion protein molecule. (Li *et al.*, 1995).

There are two potentially G·U base pairs in MiniL32, namely G10·U60 pair and U14·G53 pairs. Comparative species and compensatory mutational data suggest that an A-U pair may substitute for the U14·G53 pair, but not for the G10·U60 pair (Eng & Warner, 1991). Thus, it was initially concluded that G10 and U60 might not form a base pair, but that U14 and G53 form a wobble base pair. While the base pairing conformation of G10 and U60 is not clear, the

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[2] MBP-L30 was originally called MBP-L32, however, it has been renamed by a new nomenclature formally introduced by several laboratories (Mager et al., 1997).

importance as flanking bases of the internal loop for protein binding has been studied through mutagenesis analysis (White & Li, 1995). Studies show that G10·U60 has the highest protein affinity (10nM to MBP-L30 fusion), followed by G·A, C·C, U-A, U·C, and G·G with the other possible pairs binding very weakly. Clearly Watson-Crick pairing at this position does not favor protein binding. Finally, G10 can be replaced by inosine with only a slight reduction of binding.

Further experiments with SELEX (systematic evolution of ligands by exponential enrichment) show that the protein recognizes a motif, a 2 + 5 internal loop. Within the loop, there are no sequence variants found at position 11 and 12; position 55 can be any of the bases except G (A>C>U); position 56 must be a purine (G>>A); C is conserved at position 57; and no changes are found at position 58 and 59 (Li & White, 1997).

While much is known about the secondary structure of the RNA binding site, very limited structural information is available for the L30 protein. The only *in vivo* experiment shows that mutation of Ile43 from the highly conserved hydrophobic domain in the L30 protein leads to growth reduction (Vilardell & Warner, 1997). The mutant does not bind to its transcript, and thus no longer regulates its splicing and translation. Mutation also reduces the processing rate of the 27S pre-rRNA, the product of the second processing event of the 35S pre-rRNA that gives rise to mature 25S rRNA, resulting in an insufficiency of the60S subunit of ribosome.

1.3. NMR Methods for Protein and RNA Structural Determination

Nuclear magnetic resonance (NMR) spectroscopy has emerged as a powerful tool for solving macromolecular three-dimensional structure since the 1980s (Wüthrich, 1986; Clore & Gronenborn, 1991). Despite the relatively low upper molecular weight limit of current solution NMR technology, the driving force for the development of NMR as an alternative to X-ray crystallography was three fold. Firstly, many macromolecules do not crystallize, and even when they do there may be difficulties in solving the phase problem. Finding suitable heavy-atom derivatives is particularly difficult for nucleic acids. Moreover, significant and possibly

important functional differences may exist between structures in the crystal form and in solution, where structures solved through crystallography might suffer from the crystal packing artifacts. And finally dynamic processes ranging from the picosecond to second time scales are amenable to study by NMR. Most importantly, NMR relaxation studies can provide detailed information about molecular motions and relate the dynamics to structural recognition and functional aspects of target molecule.

With the technical advances in molecular biology and recently developed isotopic-labeling schemes, large quantities of macromolecules and reagents for *in vitro* enzymatic synthesis can be obtained. Isotopically labeled samples, in combination with powerful magnets and pulse-field gradients enable sophisticated 3D and 4D heteronuclear NMR experiment for rapid and unambiguous determination of three-dimensional structures of large molecules (up to 30,000 Dalton) using NMR spectroscopy.

1.3.1. General procedures for protein and RNA NMR

Currently, NMR structure determination relies mainly on three principle experimental variables to give geometric information. First, the chemical shift of a particular spin is directly related to its conformation and environment. Empirical surveys and theoretical calculations for proteins and peptides show that the deviations of the backbone chemical shifts from random coil values are directly related to the conformation of the peptide (Wishart & Sykes, 1994). Specific resonance assignments alone can provide a rapid and accurate mapping of a protein secondary structure. Second, interproton distances of less than 5 Å can be measured by the nuclear Overhauser effect (NOE), the intensity of which is directly proportional to r^{-6} between two proton spins. Backbone and side chain torsion angles can be calculated from the scalar coupling (*J*-coupling), the value of which is a trigonometric function of the torsion angle of two spins separated by three bonds.

After assignment of chemical shifts, approximate interproton distances and torsion angles are used for calculating an ensemble of three-dimensional structures. A number of computational strategies can be applied to locate the minimum of a target energy function, which require a combination of the satisfaction of experimental restraints without the sacrifice of covalent geometry (*i.e.* bonds, angles, planes, and chirality) and nonbonded contacts. Typical algorithms operate either in n-dimensional distance matrix followed by projection into real space (such as metric matrix distance geometry (Crippen & Havel, 1988)) or directly in real space (dynamic simulated annealing (Nilges, 1988) and restrained molecular dynamics (Nilges *et al.*, 1991)). While the distance geometric method relies on only distance information on given sets of atoms, all the real space methods require initial structures, with random arrays of atoms or structures containing correct covalent information. The quality of final structures consistent with the experimental data can be assessed qualitatively from a best fit superposition of a series of computed conformers and quantitatively by calculating the average atomic root-mean-square distribution (rmsd) of the individual structures about the mean coordinate positions.

In order to access chemical shift information, and obtain accurate experimental restraints derived from NOE and *J*-coupling information, it is essential to have individual resonance identified. In principle, sequential resonance assignments can be obtained from 1D through bond experiments such as TOCSY (TOtal Correlation SpectroscopY), and through space experiments such as NOESY (Nuclear Overhauser Effect SpectroscopY) and ROESY (Rotational Overhauser Effect SpectroscopY). With the correct resonance assignment in hand, one can then proceed to identify long-range NOE interactions between protons belonging to residues far apart in the primary sequence but close together in space, a process that yields crucial information for determining the tertiary fold of the macromolecule.

1.3.2. Isotopic labeling methods for protein and RNA

While the principles for sequence specific assignment are simple, the practice is difficult. For a small protein (less than 50 amino acids) with about 400 or less protons, spectral overlap

and chemical shift degeneracy can preclude unambiguous resonance assignments, le*t al*one quantitatively obtain distance and torsion angle information. Introduction of 2D NMR methods has improved structure determination greatly by spreading out the correlation in two ^{1}H frequency dimensions. This approach not only results in a tremendous increase in spectral resolution, but also enables ones to detect and interpret effects that would not have been possible in one dimension.

For macromolecules over 10kd, even 2D NMR cannot circumvent the problems associated with linear increases in spectral overlap and exponential decreases in efficiency of magnetization transfer through small ^{1}H-^{1}H *J*-coupling. The chemical degeneracy and resonance overlaps in the 2D NMR spectra are so severe that many pieces of critical information associated with tertiary contacting residues cannot be extracted. These effects have led to the development of isotopic labeling of macromolecules, on which acquisition of three- and four-dimensional heteronuclear spectra show improved spectral resolution through bond and through space. In addition, resultant large heteronuclear couplings also circumvent problems associated with larger linewidths in macromolecules. Thus sequence-specific assignments and unambiguous spin identification can be made with a series of triple resonance experiments.

Over the past 10 years, there have been tremendous developments in isotopic labeling scheme for both protein and nucleic acid and improvements of heteronuclear multidimensional NMR techniques. These methods necessitate the use of macromolecules uniformly labeled with ^{15}N, ^{13}C, or both. Labeled protein can be overexpressed in bacterial system, readily achieved by growing the organism in minimal medium supplemented by ^{15}N-ammonium salt and/or [^{13}C$_6$] glucose as the sole nitrogen and carbon sources, respectively. RNA oligonucleotides can be prepared by enzymatic synthesis from ^{15}N- and/or ^{13}C-labeled NTPs isolated from *E. coli* (Nikonowicz *et al.*, 1992) or *Methylophilus methylotrophus* (Batey *et al.*, 1992) grown on labeled media.

The design and implementation of higher dimensionality NMR experiments can be carried out by the appropriate combination of 2D NMR experiments (Figure 1.3.1.) (Clore &

2D

3D

¹⁵N

¹H

¹H_N

i

i+2

i+1

i+3

i slice   i+1 slice   i+2 slice   i+3 slice
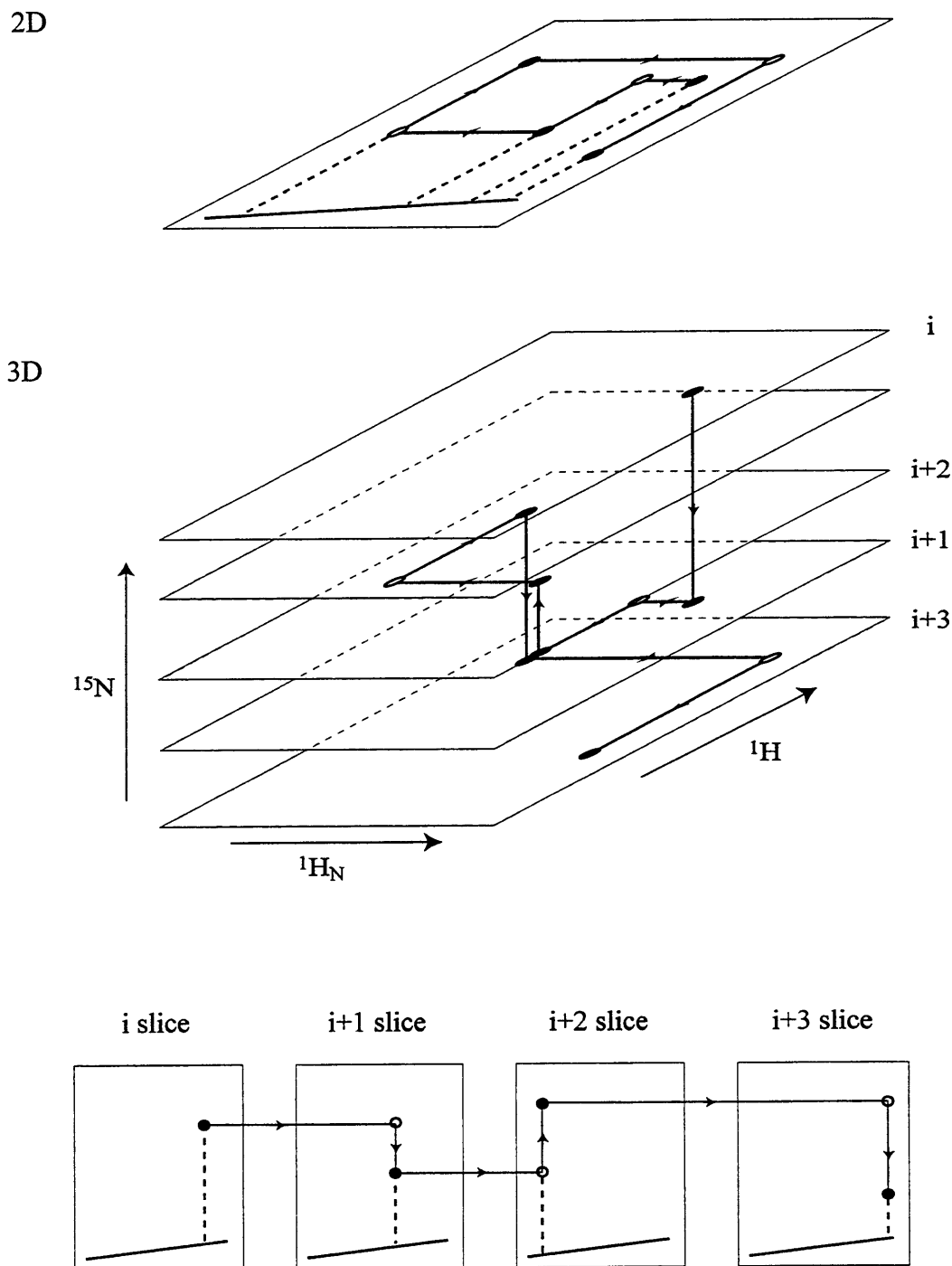
Figure 1.3.1: Schematic illustration of the progression and relation between 2D and 3D
¹⁵N-edited NOESY-HSQC spectra. The circles represent NOEs from different protons.
While assignment of these NOEs could be ambiguous in the 2D spectrum (top) because of
overlap and/or degeneracy, they can be spread into a third dimension (middle) according to
the attached heteronucleus, thus allowing unambiguous identification and sequential
connection through different 2D slices (bottom).

Gronenborn, 1991). These experiments exploit a series of large one-bond heteronuclear couplings for magnetization transfer through bonds, and together with the fact that ^{1}H nuclei are always detected, this renders these experiments very sensitive. Experiments can be devised with 3D NMR for sequential assignment that are based solely on through-bond connectivities via heteronuclear couplings (*i.e.* they do not rely on the NOE). This ability becomes increasingly important for the study of larger macromolecules. In application for protein, 3D heteronuclear triple resonance correlation experiments make use of one-bond $^{13}C'(i-1)$-$^{15}N(i)$, $^{15}N(i)$-$^{13}C_{\alpha}(i)$ and $^{13}C_{\alpha}(i)$-$^{13}C'(i)$ couplings, as well as two-bond $^{13}C_{\alpha}(i-1)$-$^{15}N(i)$ couplings, thus allow multiple independent pathways for linking the resonances of one residues with those of its adjacent neighbor. With RNA, similar triple resonance experiments have also been adapted that involve ^{13}C and ^{15}N for sugar-base correlation, base exchangeable-nonexchangeable proton correlation and, ^{13}C and ^{31}P for sequential backbone connectivity.

In summary, the yeast ribosomal protein L30 and its pre-mRNA binding site present an ideal system for studying the structure and function of a protein-nucleic acids interaction using NMR spectroscopy. The target RNA has been studied extensively through biochemistry analysis, indicating purine-rich potential non-canonical local structure(s) is (are) important for the L30 protein recognition. While less information, both biochemical and structural, is available about the protein, structure analysis using NMR spectroscopy could potentially reveal the functional aspects of the recognition, which will serve as a guideline for future biochemistry studies. Since the size of the L30 protein-RNA complex is amenable to NMR analysis, in combination of isotopic labeling technology and pulse field gradient electronics, it has been possible to obtain a high-resolution structure of this L30 complex using NMR spectroscopy. The structural elucidation of this L30 protein-RNA complex will contribute to our limited knowledge of protein-RNA interactions, in particular, how sequence and structure unite form determinants for RNA-protein recognition. Furthermore, the structural information from the L30 protein is an important piece to implement in the elucidation of the complete organization and structure of an eukaryotic ribosome.

CHAPTER 2. The L30 Protein in the Free Form

The complex of the yeast ribosomal protein L30 and its pre-mRNA binding site is a unique case for studying the structure using NMR spectroscopy. While the size of the L30 protein-RNA complex is amendable for NMR studies (*i.e.* less than 30,000 Dalton), a prudent first step to look at the individual components alone. This approach is particularly useful in NMR based analysis, since smaller size of an individual component usually translates to narrower linewidth and less chemical shift degeneracy (*i.e.* a simpler problem). By doing so, one can gains important information that might be applicable to the solution of final complex structure. In addition, comparison of the free species with the bound form shows conformational changes (if any), which may give insight into the mechanism of binding.

2.1. The L30 Protein Biochemistry

A pre-requisite of NMR experiment is to have a highly concentrated, typically millimolar, sample. For proteins, it is in general challenging to work with such high concentration, since the proteins become less well behaved. In many cases this results in precipitation, partially unfolded or aggregated states. Thus prior to conducting NMR studies of the L30 protein, it is essential to develop a L30 protein purification procedure to obtain soluble, biologically active protein.

2.1.1. Production of protein L30

To prepare millimolar NMR sample of yeast ribosomal protein L30, it is essential to acquire milligrams of protein. In early studies, L30 protein was over-expressed in *E. coli* BL21 (DE3) transfected with a pET-11a vector carrying the L30 gene under the control of a T7 polymerase promoter and an ampicillin resistance gene marker (constructed by J. Warner laboratory). However, overproduction of the protein directly in *E. coli* using the above vector

results in extensive protein aggregation. Although milligrams of protein (facilitating millimolar NMR samples) could be purified to homogeneity under urea denaturing conditions using SP cationic exchange liquid chromatography (LC) and C18 high performance liquid chromatography (HPLC), the subsequent refolding step remains problematic. Several different refolding approaches were tested in order to bring the protein back into solution from the denatured form, but none of them were successful. Gel shift analyses showed only a small fraction (less than 1%) of proteins exhibited RNA binding activity, consistent with a predominantly misfolded form (data not shown). Preliminary circular dichroism (CD) and NMR data also suggested that the refolded L30 protein adopts a random coil conformation with no significant secondary structure after attempts at refolding (data not shown).

Hence, an alternative approach to obtain NMR quantities of soluble protein was sought. Since it was clear from protein binding studies that fusion of L30 with a maltose-binding protein (MBP) stabilizes it during expression (Vilardell & Warner, 1994), and that L30 protein retains its activity after cleavage from maltose-binding protein (Li *et al.*, 1995), it might be possible to obtain large quantities of native L30 protein from the MBP-L30 fusion. However, it was not clear whether the L30 protein would maintain activity after being separated from the stabilizing MBP protein (which was not done in the previous studies (Li *et al.*, 1995)), and whether it alone would be soluble at millimolar concentration. These uncertainties were explored, and a new purification procedure to produce near millimolar concentrations of active L30 protein for NMR structural studies was established.

2.1.1.1. Cell growth

Unlabeled protein: *E. Coli* strain JM109 was chosen to host plasmid pMalc-L30 (Vilardell & Warner, 1994) (provided by Dr. Susan White from Bryn Mawr College) for over-expression of MBP-L30 fusion protein. Unlabeled protein was prepared from cells grown in LB media (15 g yeast tryptone, 5 g yeast extract, 5 g NaCl) (Sambrook *et al.*, 1989). Briefly, a single *E. coli* colony was inoculated into 5 ml LB media containing 100 mg/L ampicillin for overnight growth at 37°C. Cells from one ml of this culture were pelleted, resuspended in fresh LB media, and

transferred to a two liter Fernbach flask containing one-liter LB media and 100 mg/L ampicillin. The culture was grown in an incubator-shaker at 37°C and 250 rpm. When the cell density reached 10^8 cells/ml ($OD_{600} \geq 0.6$), the culture was shifted to 25°C for protein expression, with the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). In the mean time, an additional 100 mg of ampicillin was also supplemented into the culture. This was necessary for two reasons: β-lactamase is secreted into the media and metabolizes ampicillin, and higher cell density may result in the loss of ampicillin-resistance plasmid from the cells. After four hours of induction, the cells were harvested by centrifugation at 6000 *g* for 10 minutes, and the pellets were stored at -80°C. Typically, one liter of LB media yields about 6 grams of wet packed cells.

Isotopically labeled protein: Using the same cell strain, glucose-based minimal media instead of LB media was chosen for cell growth in the preparation of ^{15}N and/or $^{13}C/^{15}N$ labeled protein. The complete minimal media was prepared in the following manner. 3.0 g KH_2PO_4, 12.8 g $NaH_2PO_4 \cdot 7H_2O$ and 0.5 g NaCl were added into one liter distilled water, and autoclaved for 30 minutes. When the solution cooled to room temperature, the following filter-sterilized solutions were added: 2 ml 0.35 g/ml $(NH_4)_2SO_4$, 20 ml 20% D-Glucose, 1 ml 1 M $MgCl_2$, 1 ml 1 M $MgSO_4$, 1 ml 1000× trace metal supplements (750 μg $CaCl_2 \cdot 2H_2O$, 30 mg Na_2EDTA, 25 mg $FeCl_3 \cdot 6H_2O$, 240 μg $CuSO_4 \cdot 5H_2O$, 180 μg $MnSO_4 \cdot 5H_2O$, 27 μg $ZnSO_4 \cdot 7H_2O$, 270 μg $CoCl_2$), 1 ml 1000× vitamin mixtures (2 ng biotin, 2 ng folic acid, 5 ng thiamine, 5 ng calcium pantothenate, 0.1 ng vitamin B12, 5 ng nicatinamide, 5 ng riboflavin per ml), and 1 ml 100 mg/ml ampicillin. Uniformly ^{15}N labeled protein was expressed in minimal media containing 0.70 g/L $(^{15}NH_4)_2SO_4$ as the sole nitrogen source. Uniformly $^{13}C/^{15}N$ labeled protein was expressed in minimal media containing a slightly higher concentration of $(^{15}NH_4)_2SO_4$ (1.0 g/L), and with less $^{13}C_6$-glucose (1.0 g/L) as the sole nitrogen and carbon source, respectively. The cell growth procedure was similar to that of the LB medium, except that the doubling rate in minimal media was twice as slow, and the final yield was lower. Typically, about 3 - 4 grams of ^{15}N labeled and 2 - 3 grams of $^{13}C/^{15}N$ labeled cells are obtained from a one liter minimal media growth.

2.1.1.2. Protein purification

<u>MBP-L30 fusion protein purification:</u> About 5 grams of cells were thawed on ice and resuspended in 50 ml buffer containing 20 mM Tris•HCl (pH 7.0), 200 mM NaCl, 1 mM EDTA and 5 mM DTT. After the addition of 50 µl 200 mg/ml PMSF and 10 µl 40 unit/µl DNase, cells were lysed by sonication (20 pulses of 30 s duration with 2.5 min rest in between) with a 550 Sonic Dismembrator (Fisher Scientific). Nucleic acids and proteins that are negatively charged were precipitated with the addition of 750 µl of 10% polyethylene imine (pH 8.0) and moderate stirring on ice for 30 minutes. After centrifugation (10,000 g) to pellet the debris, the supernatant containing the target MBP-L30 fusion protein was loaded onto a 50 ml Carboxyl Methyl cellulose (CM) column (TOYOPEARL). The column was washed with lysis buffer (about 100 ml) until the UV absorbance of the flow through dropped to baseline level. A salt gradient varying from 200 mM to 500 mM NaCl was used to elute the protein. MBP-L30 fusion protein eluted around 300 mM NaCl. Because the CM column selectively purifies protein based on positive charges, it was observed that some RNases purified with the target protein. Therefore, the MBP-L30 protein was further purified on an amylose affinity column (New England Biolabs) to remove any RNase contaminants. The CM column fractions containing MBP-L30 protein were directly loaded onto a 30 ml amylose column, and washed extensively with freshly prepared lysis buffer (20 mM Tris•HCl (pH 7.4), 200 mM NaCl and 1 mM EDTA). The MBP-L30 protein was eluted using a column buffer containing 10 mM maltose. The protein purity was determined on a 10% SDS PAGE gel, and the removal of RNase contamination was monitored through an RNase activity assay (data not shown).

<u>Factor Xa cleavage:</u> The fusion protein was designed such that the MBP is linked to the L30 protein by a Factor Xa site, and hence free L30 can be produced by the cleavage reaction as described hereafter. About 30 mg of MBP-L30 protein was concentrated in a Centriprep-10 device (Amicon Inc.) to a final volume of 6 ml. The cleavage reaction was initiated by the addition of 12 µl 1 M CaCl$_2$ and 150 units of 1 mg/ml Factor Xa (NEB) (Nagai & Thogersen, 1984). About 80-90% of full length L30 was cleaved from MBP right after the engineered recognition site (Ile-Glu-Gly-Arg) in 18 to 36 hours at 4°C.

L30 isolation: The separation of L30 protein from the proteolytic cleavage mixture was achieved through CM column chromatography based on the charge differences of L30, the MBP-L30 fusion and MBP. L30 protein has the greater net positive charge at pH 7.0, and can thus be separated away from MBP and the MBP-L30 fusion using a salt gradient. A 200 ml salt gradient from 200 mM to 1 M NaCl concentration prepared in 10 mM sodium phosphate buffer (pH 7.0) was used to elute L30 from the CM column (Figure 2.1.1). This purification procedure gives an average yield of approximately 5 mg of homogeneous L30, as quantified by either a BioRad protein assay or UV absorbance at 280 nm ($\varepsilon_{280} = 8976$ M^{-1}cm^{-1}). The identity and integrity of the protein was confirmed by electro-spray mass spectrometry. The purified L30 shows a single molecular mass of 11295.1, which is comparable to the calculated mass of 11294.4 at pH 4.0. The activity of L30 protein was verified by an *in vitro* RNA binding assay.

In summary, the protein expression and purification of L30 using fusion with the maltose-binding protein described above, though relatively time consuming, is an efficient and straightforward procedure. Detailed below are a few precautions that need be taken in order to generate a protein sample with both the high quality and purity required for structural studies.

First, the expression of soluble MBP-L30 fusion protein is sensitive to IPTG concentration and the temperature at which the induction of protein expression occurs. Relatively lower IPTG concentration (0.4 to 0.5 mM) and lower temperature (25°C) in this case yield more soluble fusion protein when compared to typical conditions (1 mM and 37°C, respectively). A good expression can be judged from direct observation of the lysate on a SDS-PAGE gel. Concomitant with the SDS-PAGE observation, the appearance of the cell lysate also gives clues to success. A translucent lysate suggests soluble protein, while an opaque lysate is an indication of severe aggregation from protein expression. Caution also must be taken for expression of isotopically labeled protein, because of the slow growth in minimal media and secretion of β-lactamase into the media that can lead to partial plasmid loss in *E coli*. Additions of ampicillin during the protein expression helps to increase the protein yield.
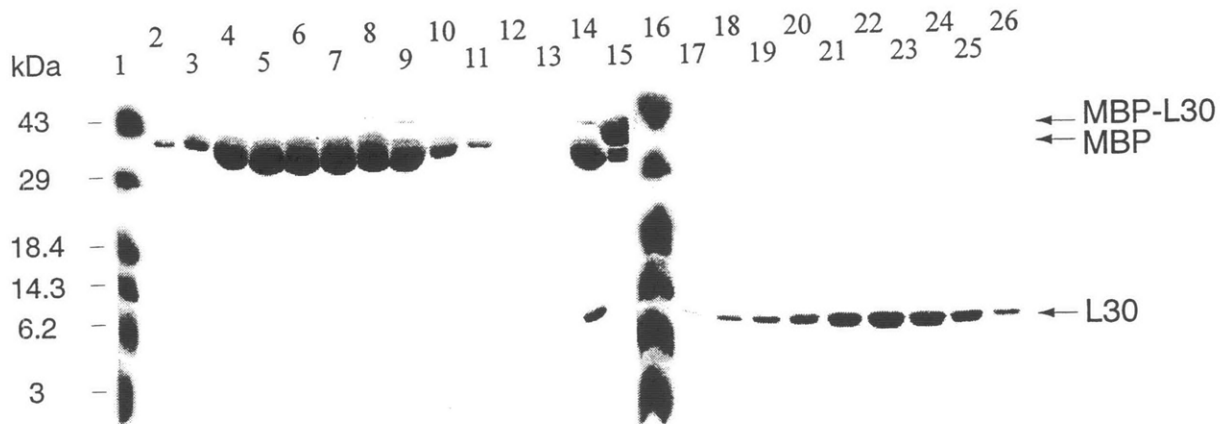
Figure 2.1.1: Protein separation profiles from a CM column purification viewed on 16% SDS PAGE gels: lanes 1 and 16 are molecular mass markers; lanes 2-11 show the CM column flow-through containing the majority of MBP with trace MBP-L30 fusion; lane 14 shows the Factor Xa protease cleavage mixture of MBP-L30; lane 15 shows a mixture of the MBP-L30 and MBP from an amylose column purification; lanes 17-26 show the purified L30 protein after a salt gradient elution.

Second, a good separation of target protein from cellular nucleic acids is a critical step in L30 protein purification. Since L30 protein is an RNA binding protein, some nonspecific interactions with cellular nucleic acids is expected. However, nucleic acids that co-purify will not only interfere with accurate determination of protein concentration (by UV absorbance at 280 nm), but also affect the quality of NMR spectra. Polyethylene imine (PEI) precipitation of nucleic acids after cell lysis turned out to be an important step for the protein purification. PEI is a basic linear polymer of molecular weight 30,000-90,000 that forms precipitates with negatively charged macromolecules under low ionic strength, and it has been widely used for nucleic acid binding protein purifications (Burgess, 1991). During the initial stages of the MBP-L30 fusion protein purification, addition of 0.1% PEI at pH 7.0 in the cell lysis buffer can significantly reduce the levels of nucleic acids and most acidic proteins. The latter includes the maltose binding protein, which is a side product of fusion protein degradation.

The above native L30 protein purification is a better approach than a denaturing preparation. Although it is straightforward to directly express large quantities of L30 protein in *E. coli* as inclusion bodies, attempts to subsequently refold the protein were not successful. There are at least three different experimental observations, as described hereinafter, supporting a less than ideal refolded protein. First, although some proteins (*e.g.* S15 (Batey & Williamson, 1996), U1A (Nagai *et al.*, 1990)) can be refolded upon binding to their target RNA, this does not hold true for the L30 protein. Judging from the native gel shift assay, less than 1% of the refolded protein gains binding activity to MiniL32 RNA. Second, the ^{1}H-^{15}N HSQC correlation spectrum of refolded protein displays very narrow chemical shift dispersion (about 1 ppm in the ^{1}H dimension) around 8.3 ppm, which is a characteristic of a molten globule and/or random coil conformation. This was also evident from NOESY experiments as there were only a few NOE cross peaks in the whole spectrum (typically folded proteins show many NOE cross peaks). Third, while the CD wavelength spectrum at neutral pH indicated the presence of some secondary structure in the refolded protein, the thermal melt showed a shallow and elongated transition, indicative of an aggregate or molten globule (data not shown).

On the contrary, L30 protein co-expressed as a maltose-binding protein fusion remains soluble under the aforementioned optimal native expression conditions, and purification of the MBP-L30 fusion on cationic and affinity columns renders homogenous fusion protein with optimal RNA binding affinity. Subsequent cleavage with Factor Xa to generate L30 protein also displays similar RNA binding activity. Finally, it was possible to concentrate this protein into half millimolar concentration for further NMR characterization.

2.1.2. *In vitro* RNA binding assay

2.1.2.1. Dissociation constant (K_d) determination

The purified MBP-L30 fusion and L30 protein were subjected to an RNA binding assay in order to ascertain the protein activity and stoichiometry of binding. In published results (Li *et al.*, 1995), most RNA binding affinity and specificity assays were conducted using the MBP-L30 fusion, and verification that the sole RNA binding activity comes from L30 protein was tested in the Factor Xa mixture without further separation. During the course of the present protein purification, both purified MBP-L30 fusion protein and purified L30 protein from cleavage were tested for RNA binding. Similar to the published assays, the buffer used in this study contained 30 mM Tris·HCl (pH 8.0), 75 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 50 ng/μl bovine serum albumin (BSA) (New England Biolabs), 40 ng/μl tRNA (Sigma), and 0.2 unit/μl RNase inhibitor (Promega). Zero to 5000 ng/μl of MBP-L30 fusion protein or 0 to 1000 ng/μl of L30 protein was titrated into less than 0.01 nM 5' end [γ-P^{32}] labeled RNA ($<< K_d$) to achieve pseudo first order binding. Briefly, 0.01 nM 5' [γ-P^{32}] labeled RNA was resuspended in 30 mM Tris·HCl, 75 mM KCl and 10 mM DTT, denatured at 65°C for 1 minute, and flash cooled on ice to induce RNA monomer formation. After the labeled RNA was equilibrated on ice for 5 min, 1 μl 80 ng/μl tRNA and 0.2 μl RNasin were added per 10 μl solution. The mixture was aliquoted as 10 μl fractions into 500 μl siliconized Eppendorf tubes and kept at room temperature. In the mean time, a series of two fold dilutions were used to prepare L30 protein with concentrations from 2000 to 1.95 ng/μl (or MBP-L30 fusion from 10000 to 9.8 ng/μl) in binding buffer (30 mM

Tris·HCl, 75 mM KCl, 10 mM DTT and 100 ng/μl BSA). The binding reaction was initiated by

mixing 10 μl of protein (with varies concentration) into 10 μl of RNA (constant concentration),

incubated at room temperature for 20 min, and immediately subjected to an electrophoretic gel

shift experiment. Typically, 10 μl of each binding mixture was loaded onto 10% (w/v) native

gels (29 to 1 ratio of acrylamide to bisacrylamide) with 0.5× TBE running buffer at 200 V/20 cm

in a 4°C cold room. The resulting labeled bandshift data were quantified using a

phosphorimager (Molecular Dynamics, Inc.). All binding affinity data were fitted to hyperbolic

binding isotherms in the Pro3.0 Igor program (Wavemetrics).

2.1.2.2. Stoichiometry determination

The stoichiometry experiments were carried out similar to K_d determination with a slight

modification. Instead of using trace amounts of RNA to achieve pseudo first order binding, the

concentration of RNA in the assay was raised above K_d (~14 nM). About 300 nM unlabeled

RNAs were titrated with increasing concentrations of L30 protein (or MBP-L30 fusion) ranging

from 10 nM to 10 μM. The fractions of bound RNA were traced by a small amount of 5' [γ-P^{32}]

labeled L30 RNA added to the unlabeled RNA in the binding mixtures.

Freshly purified L30 protein and the MBP-L30 fusion have dissociation constants of 4

nM and 12 nM, respectively (Figure 2.1.2), which are similar to the reported values (10 ± 3 nM

and 3 ± 1 nM, respectively) (Li *et al.*, 1995). At least five- to ten- folds larger K_d values were

observed during the earlier purification when inactive protein was copurified. Decreasing

binding activities were also observed for both L30 protein and the MBP-L30 fusion with

prolonged storage, with a much faster decay rate for L30 protein (half life less than couple of

weeks). The activity loss can be quantified in a stoichiometry assay. A 1:1 stoichiometry of

protein to RNA is typical with freshly prepared L30 protein and MBP-L30 fusion, however, if

the ratio is any larger than 1:1 ratio, this indicates a potential loss of RNA binding activity.

Because of the differences in the long-term stability, the MBP-L30 fusion has been used in the

majority of K_d studies for testing different RNAs.

a.   b.

MBP-L30 / RNA

L30 / RNA

RNA

1   1516   30
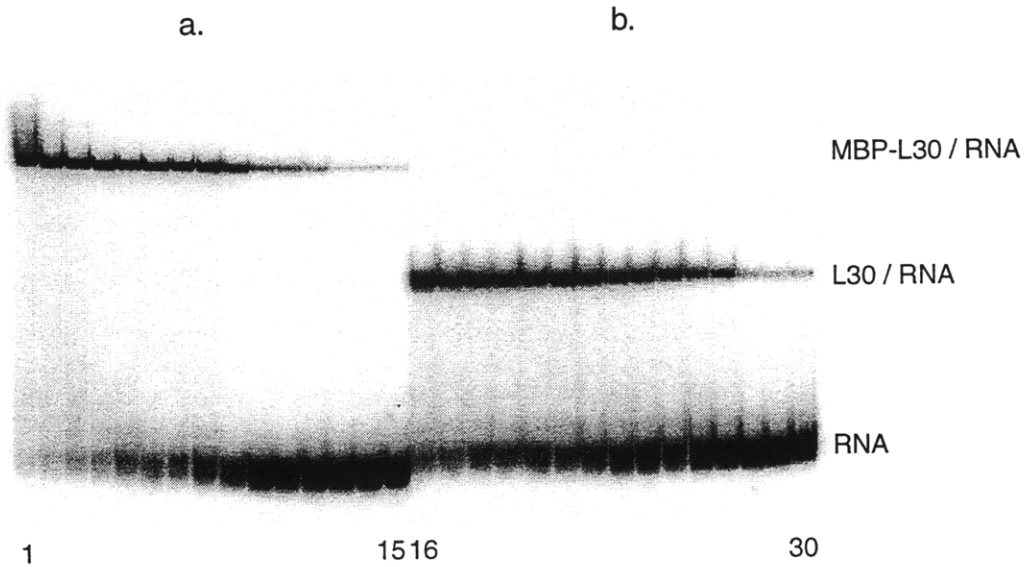
Figure 2.1.2: Gel shift assay of **a**, MBP-L30 fusion and **b**, L30 protein binding to L30N RNA. The 10% native polyacrylamide gel was run at 4°C. From left to right in lanes 1 to 15 the MBP-L30 concentration is 2000.0, 1000.0, 500.0, 250.0, 125.0, 62.5, 31.7, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.3, and 0.1 μM; in lanes 16 to 30 the amount of L30 protein is 8000.0, 4000.0, 2000.0, 1000.0, 500.0, 250.0, 125.0, 62.5, 31.7, 16.0, 8.0, 4.0, 2.0, 1.0, and 0.5 μM.

2.1.3. Circular dichroism

2.1.3.1. Secondary structure estimation

The α-helical and β-sheet contents of the L30 protein in the absence and presence of RNA were measured by circular dichroism, using either Aviv 60 DS or Aviv 292 SF spectrophotometers. Scans from 200-300 nm (0.5 nm steps, 15 second averaging time) were acquired (0.1 cm path length) on 400 μl 25 μM samples in 10 mM potassium phosphate buffer, pH 6.5 at 4°C. While potassium phosphate buffer was used for background correction of the free form protein signal, free RNA in the same buffer was used for the complex. This of course assumes no significant secondary structure changes upon binding with the RNA upon binding. The signal output, millidegree (m°), was converted to molar ellipticity ([θ]) via the following equation:

$$[\theta] = 100 * m°/(l\ c\ N_{res})\tag{2.1}$$

where l is the path length of the cell (in cm), c is the concentration of the sample (in millimolar), and N_{res} is the number of residues in the protein.

The molar ellipticity at any fixed wavelength between 190 nm to 300 nm can provide a rough estimation of the secondary structure content of a given protein. In particular, the α-helix, β-sheet and coil forms of proteins each has its characteristic CD spectrum. The CD signal of a pure α-helix has two minimums at 222 and 206 nm, and a large positive band at 190 nm, which represent the n-π*, π-π$_{\parallel}$*, and π-π$_{\perp}$* transitions; a β-sheet has a minimum at 216 nm, and two small positive bands between 225 and 250 nm; and coil has a minimum at 223 nm and a maximum around 200-202 nm. These characteristic bands can be used to qualitatively predict the predominant secondary structure in a protein. This molar ellipticity at any fixed wavelength can be calculated from the percent helix and sheet content according to the following equation:

$$X = f_{H}\,X_{H} + f_{\beta}\,X_{\beta} + f_{R}\,X_{R}\tag{2.2}$$

where X is the mean residue ellipticity, [θ], the X_H, $X_β$, X_R are the reference values for pure α-helix, β-sheet and coil form (R) (Chen *et al.*, 1974).

The CD spectrum of the bound form of L30 protein closely resembles that of the free form (respective **b** and **a** in Figure 2.1.3). The spectra of both forms showed the presence of a mixture of α-helical and β-sheet conformation, with the bound form protein showing about 25% more negative signal around the lowest minimum (208 nm). Detailed analysis of the CD spectra using equation (2.2) gave an estimation of 50% α-helix, 25% β-sheet and 25% coil in the free form L30 protein. This information can be corroborated with analyses carried out in NMR studies. No estimation was made on the bound form L30 protein. Even though the additional signal could be explained as a more ordered protein secondary structure, potential contribution from the large absorbance of an RNA at 208 nm could influence the signal

2.1.3.2. Thermal denaturation

While the CD spectra provide a rough estimation of secondary structure of the L30 protein, circular dichroism thermal melts can be used to study the temperature dependent stability of the protein in the absence and presence of the RNA. Data points monitored at 218 nm from 4 to 88°C (2°C temperature step, 1 min equilibration, and 30 sec average time) were taken on the same samples used in the CD wavelength scans. The molar ellipticity ([θ]) was fitted to a hyperbolic curve in Igor to derive the melting temperature.

As can be seen from the inserts in Figure 2.1.3, the free and bound L30 proteins have dramatically different thermal stabilities, despite sharing a great similarity in the secondary structures. While the free form protein has a shallow and elongated melting curve with an irreversible denaturation around 45°C, the bound form has a sharp and irreversible melting transition at a higher temperature around 68°C. The CD-monitored thermal denaturation of free form protein suggests that although the secondary structure elements are present, the protein can only adopt a quasi-folded (or a molten globule) conformation at low temperature, and the tertiary interactions are highly unstable. Contrary to the free protein, the RNA-bound L30 protein has significantly better thermal stability at temperatures below 60°C. Furthermore, a cooperative
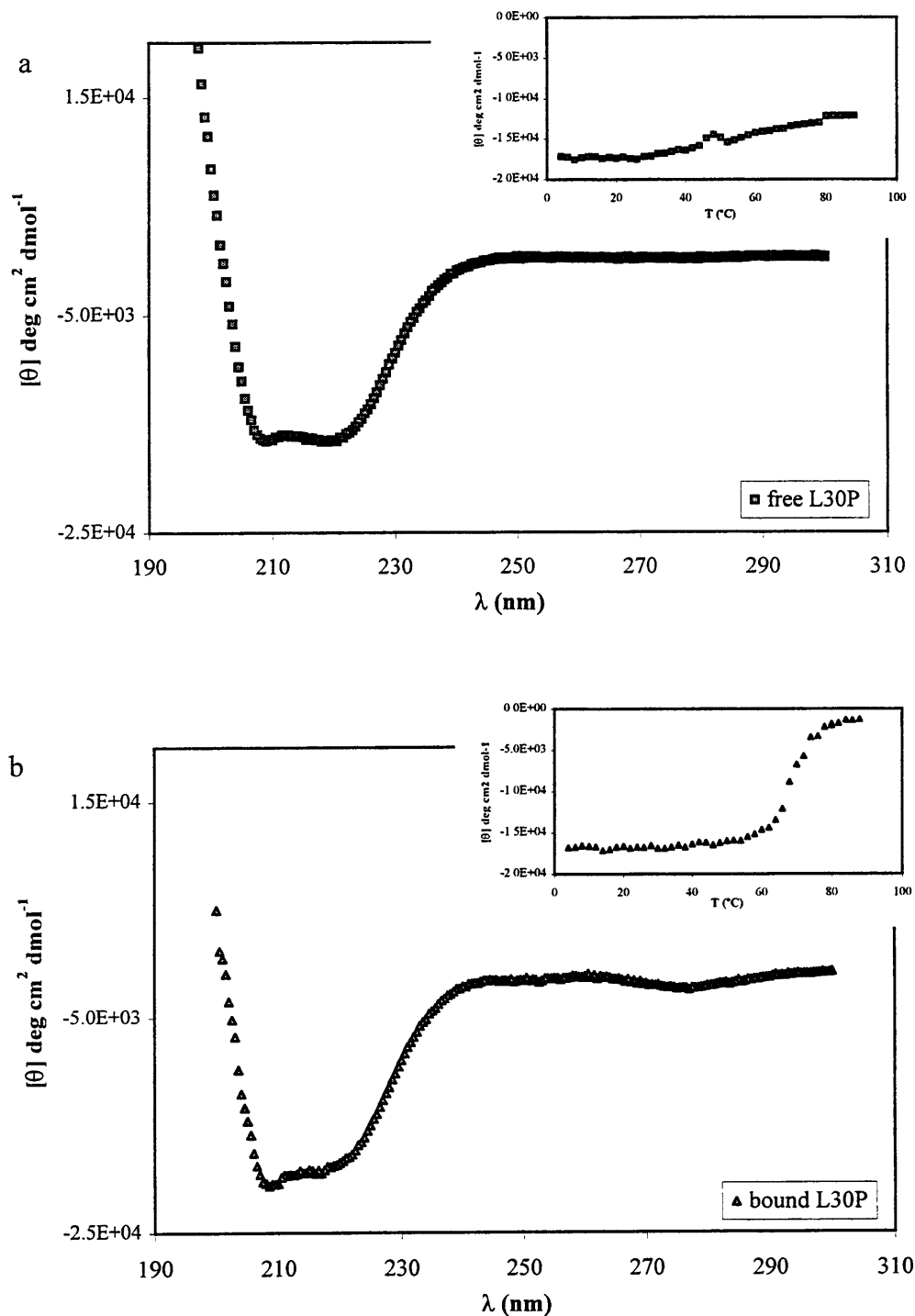
Figure 2.1.3: A comparison of the CD spectra of **a**, the free form L30, **b**, the RNA bound L30 protein. The concentrations were approximately 25 μM. The solution conditions are 10 mM potassium phosphate buffer, pH 6.5. The insets to **a** and **b** show the thermal denaturation of the proteins monitored at 218 nM.

thermal denaturation transition indicates a completely folded structure and/or stabilization of tertiary structures in the presence of RNA. The studies of the temperature-dependent stabilities of free and bound form L30 proteins were important for assisting the subsequent NMR characterization.

2.2. NMR of L30 Protein in Free Form

2.2.1. Optimization of NMR conditions

The first step in protein NMR spectroscopy is the optimization of sample conditions to achieve the most folded structure and hence the best dispersed spectra. At the onset, one may think that it would be best to have maximum sample concentrations, but this may not be true. Not all proteins are stable at high concentration, therefore an optimization must be drawn for each protein. In addition to the sample concentration, other factors also affect the NMR signals. The sensitivity of a one-dimensional experiment is proportional to:

$$S/N \sim N \, \gamma_{exc} \gamma^{3/2}_{det} \, B_0^{3/2} \, (NS)^{1/2} \, (T_2 \, / \, T)$$

where N is the number of molecules in the active sample volume, γ_{exc} is the gyromagnetic ratio of the excited spin, γ_{det} is the gyromagnetic ratio of the detected spin, B_0 is the static magnetic field, NS is the number of scans. T_2^{-1} is the homogeneous line width, and T is the temperature in Kelvin. As can be seen, besides sample concentration, NMR signal is also directly related to acquiring temperature and transverse relaxation T_2 time (Cavanagh *et al.*, 1996). The transverse relaxation time can be prolonged with reduced sample viscosity using higher temperature or less amount of sample. With a given amount of sample, buffer pH, salt concentration and temperature also affect the intrinsic solubility of the protein, therefore the necessity to ascertain the optimal sample condition can be seen.

There are various methods to monitor protein folding by NMR. These vary from a simple proton 1D to heteronuclear correlation experiments. One of the best ways to achieve this is to monitor the ^{1}H-^{15}N correlations. Both the backbone ^{15}N and amide proton resonances are highly sensitive to the environment, and a well dispersed ^{15}N-HSQC spectrum is a direct indication of a well-folded protein. Therefore, a series of ^{15}N-HSQC spectra were recorded for the uniformly ^{15}N-labeled L30 protein at various sample conditions to monitor the best NMR signal.

The optimization of sample condition was conducted by screening temperature, pH and salt concentration of the sample using ^{1}H-^{15}N FHSQC (Mori *et al.*, 1995) spectra recorded on the MIT Francis Bitter Lab 501 MHz instrument. Consistent with the observations during protein purification and results from CD thermal melt analysis, the instability of uncomplexed L30 protein is also directly observed in NMR experiments with temperatures above 15°C. In addition to temperature, the stability of L30 protein was also sensitive to pH, salt and sample concentration. Specifically, a 0.7 mM protein sample undergoes significant aggregation at the following conditions: temperature above 15°C, pH above 8 or below 5.5, low salt (below 200-300 mM NaCl). Because of this limitation, all the NMR experiments used to characterize the free form protein were recorded at 10°C with less than 0.7 mM protein sample in 300 mM NaCl, 0.02% NaN$_3$, 0.1 mM EDTA and 2.0 mM Tris·HCl (98% deuterated) buffer, pH 6.5. For H$_2$O samples, 5% D$_2$O was included for deuterium locking; for D$_2$O samples, the samples were lyophilized, exchanged with 99.8% D$_2$O (Cambridge Isotope Laboratories, Cambridge, MA) twice, and redissolved in 99.996% D$_2$O (Isotech, Inc.). Even with the above optimal condition (*i.e.* low sample concentration, low temperature and high salt), obvious protein aggregation was observed over a two-week period. This clearly limited the application of many of the NMR experiments.

The free form L30 protein did indeed give a high quality NMR spectra, as can be seen from the ^{1}H-^{15}N HSQC spectrum acquired at 10°C on a 0.5 mM sample, which correlates amide protons to its attached nitrogen (Figure 2.2.1). The good backbone resonance dispersion clearly suggests formation of distinct secondary structures and possible tertiary contacts within L30
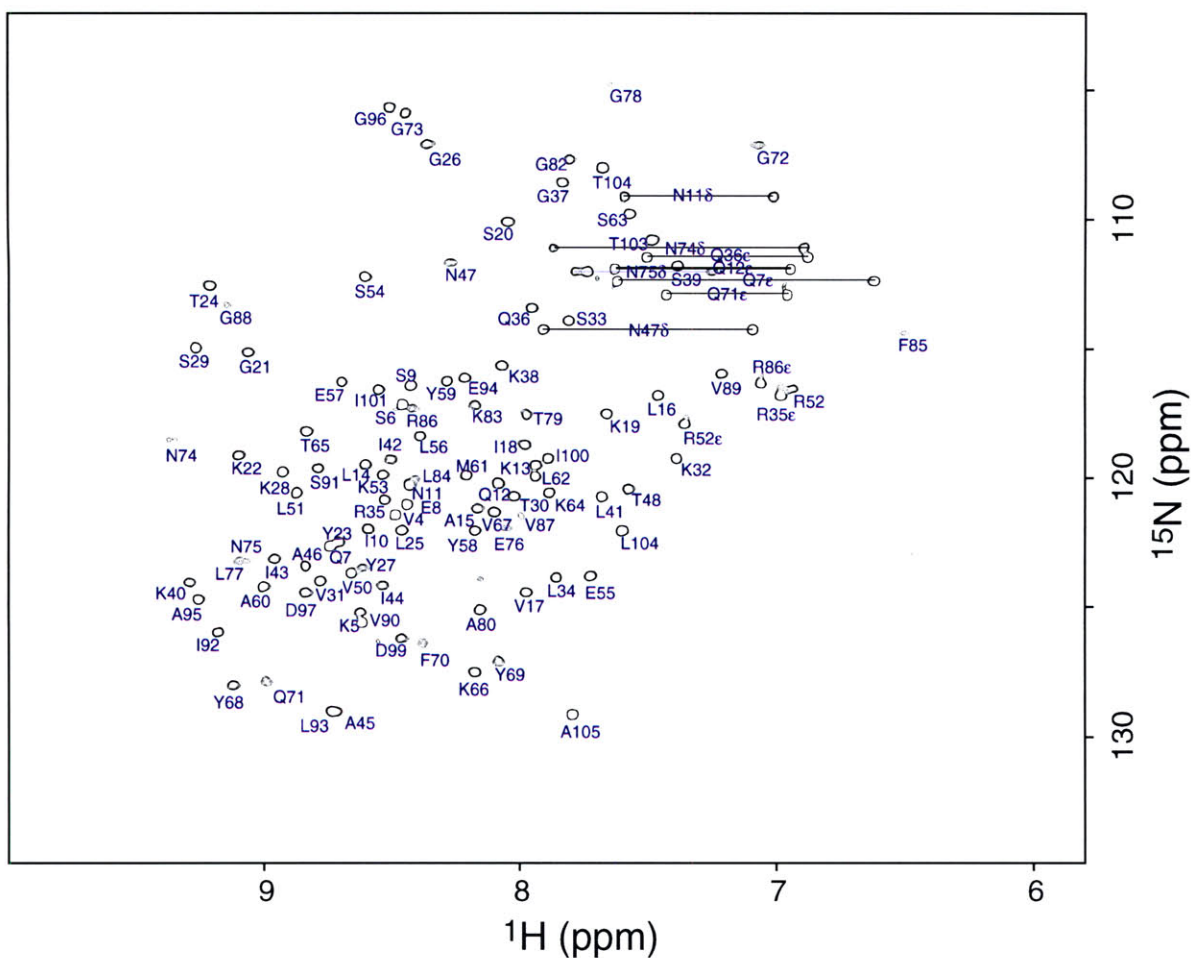
Figure 2.2.1: ^{1}H-^{15}N HSQC spectrum of the L30 protein in the free form at 10°C. The peaks are labeled with their assignments, with the one-letter code for the amino acid type followed by the sequence position. The side chain amides for Asn and Gln are connected with horizontal lines and labeled with their assignments and side chain positions with Greek letters. Arg ε side chain peaks are folded in this spectrum.

protein. Similar to circular dichroism analysis, chemical shifts in the NMR spectra also provide a general measurement of secondary structures in protein. It has been demonstrated for all amino acid residues that secondary structure formation induces very specific and significant chemical shift changes (Wishart *et al.*, 1992). The method for estimating the rough secondary structure content in protein was based on these following empirical observations: 1) The number of residues in β-strands is proportional to the number of H_α resonances between 4.85 and 5.90 ppm. 2) The number of residues in the coil conformation is proportional to the number of backbone amide resonances between 8.20 and 9.00 ppm. 3) The number of residues in helices is proportional to the number of non-glycine H_α resonances between 3.40 and 4.10 ppm (Wishart & Sykes, 1994). A simplified analysis of the secondary structure of L30 protein was carried out on the basis of the amide backbone resonances in the ^1H-^{15}N HSQC spectrum. This gave an estimate that about 35% residues are in coil conformation, with the rest either in β-sheet or α-helix conformations. The percentage of secondary structure components is close to the result estimated from CD data, with about 25% of residues in coil conformation, and 75% in either β-sheet or α-helix conformations. It is also clear from the ^1H-^{15}N HSQC spectrum that the protein adopts a folded conformation in solution at close to millimolar concentration that allows further structural analysis.

2.2.2. NMR studies and resonance assignment

The first step towards structure determination by NMR is the sequential assignment of all the resonances in the spectra. L30 protein has a molecular weight of about 11 kDa, thus it is expected that the assignments can be made using the conventional NOESY, COSY and TOCSY types of 2D experiments. But a surprisingly high degree of resonance overlap and chemical shift degeneracy has been seen in the present case, which makes accurate interpretation somewhat difficult. Therefore it is necessary to utilize heteronuclear 2D/3D based assignment. In these experiments, the degeneracy of the proton resonances can be alleviated by the distinct chemical shifts of the heteronuclei, namely the carbons and the nitrogen. Furthermore, it is possible to

spread the proton 2D spectrum into a third dimension according to the chemical shift of the directly bonded heteronuclei.

2.2.2.1. Assignment of the exchangeable protons:

The ^{1}H-^{15}N HSQC spectrum provides the fingerprint for the protein, as the amount of peaks is directly correspond to the number of amino acids. One might expect to see one backbone ^{1}H$_N$-^{15}N peak correspond to each of all the amino acids (except the prolines). Besides this, the spectrum also displays the ^{1}H$_N$-^{15}N correlations for the side chains. The L30 protein contains 104 amino acid residues, with two prolines (Pro) that have no backbone amide protons, and four glutamines (Gln), four asparagines (Asn) and three arginines (Arg), all having side chain amide protons. Excluding the N-terminal residue Ala2, of which the amino group is expected to exchange with solvent, and thus generally not observed, one might expect to observe a total of 120 peaks corresponding to 101 backbone amide peaks, eight pairs of side chain amide and three Arg side chain peaks. By careful analysis of the spectrum acquired at 10°C at pH 6.5 (Figure 2.2.1), and along with the HSQC spectra at different temperatures (10, 15, 20 and 25°C) and pH (5.0, 5.5, 6.0), it was possible to identify 116 ^{1}H-^{15}N resonances, which were further identified as belonging to 97 backbone amide peaks, 8 pairs of side chain amides for Asn and Gln, and 3 Arg side chain amides. The smaller number of observed peaks, and the fact that some of the peaks exhibited broad linewidths and were only just visible at the contour level, suggest a possible regional conformational exchange broadening in the free form of the protein. Nevertheless, since most resonances display good dispersion and sharp linewidth, it is possible to carry out further specific resonance assignment.

In order to map out the sequential connectivities, 3D ^{15}N edited experiments were acquired. The assignments were made based on heteronuclear 3D NOESY and TOCSY experiments. The former leads to sequential assignments, and the latter helps in spin system identifications. More specifically, TOCSY transfers occur within the *J*-correlated protons (*i.e.* intraresidue), whereas nuclear Overhauser effects (NOEs) are observed between protons that are close in space (typically < 5 Å), which occur by dipolar interactions. NOE patterns in the protein

spectra are also the signature of the secondary structure. Typically, in an ^{15}N edited NOESY spectrum, characteristic sequential NOE patterns are observed between H_N-H_N resonances of the adjacent residues, and H_N -H_α resonances of approached residues i and i-3, and residues i and i-4 for an α helix; whereas β sheets show characteristic H_N -H_α resonances between residue i and i+1, and long-range backbone H_N-H_N cross peaks.

A ^{15}N NOESY-HSQC experiment with 150 ms mixing time was acquired at Bitter 501 MHz instrument, using WALTZ (Shaka *et al.*, 1983) decoupling during acquisition. The ^{15}N transmitter was set at 116 ppm, centered at the backbone amide resonance, and the ^{1}H transmitter was set at 4.93 ppm for water suppression. A data set with 512 complex points in t_3 (^{1}H), 128 complex points in t_2 (^{1}H) and 32 complex points in t_1 (^{15}N) was acquired with 6000 Hz spectral widths in both t_3 (^{1}H) and t_2 (^{1}H) dimensions, and 1600 Hz sweep widths in the t_1 (^{15}N) dimension. A total of 32 scans were acquired for each increment.

Before analyzing the NOESY spectra, it is essential to obtain the spin system identification, that is identifying each amino acid first by correlation of the backbone to its side chain. This was accomplished by an ^{15}N TOCSY-HSQC (Grzesiek *et al.*, 1992) recorded on Varian Inova 600 MHz instrument, using 12 kHz for the spin-lock and a 50 ms mixing time (to maximize ^{1}H$_N$-^{1}H$_\alpha$ correlation). The three dimensional spectrum was acquired with 512 × 128 × 32 complex points with 7000 Hz spectral widths for both t_3 (^{1}H) and t_2 (^{1}H) dimensions, and 2000 Hz for the t_1 (^{15}N) dimension.

An almost complete assignment for the amides of the L30 protein was made primarily with the combination of the above two experiments using the standard approach (Wüthrich, 1986). Briefly, once the ^{15}N strips were identified based on the TOCSY correlations, the ^{15}N strips from the NOESY-HSQC 3D spectrum were aligned to locate the positions of intraresidue NOEs peaks. Once the intraresidue peaks are identified, the corresponding interresidue NOE peaks are used for sequential connection. One advantage in the 3D NOESY-HSQC spectrum is that the $H_N(i)$-$H_N(i+1)$ NOEs occur twice in a symmetric manner that allows the identification of the adjacent H_N pairs. In many cases, the strongest NOEs for any given amide proton are

invariably the sequential $H_\alpha(i)$-$H_N(i+1)$ or $H_N(i)$-$H_N(i+1)$ connection. Long-range NOEs (*i.e.* interstrand H_N-H_N or H_N-H_α connections) are invariably of lower intensity. The α-helix or β-sheet stretches in the protein were identified according to the characteristic NOE and TOCSY patterns. For example, shown in Figure 2.2.2, residues 14 - 17 occur as a regular α-helix, and hence are manifest in several ways in the 3D NOESY-HSQC and TOCSY-HSQC spectra: showing strong amide protons $H_N(i)$ - $H_N(i+1)$ peaks, medium intraresidue H_N-H_α, and interresidue $H_N(i)$-$H_\alpha(i-1)$ peaks, and medium $H_N(i)$ - $H_\alpha(i-3)$ peaks in the NOESY spectrum and weak H_N -H_α scalar correlation in TOCSY spectrum. In contrast, residues 90-93 that adopt a β-sheet conformation have intense H_N-H_α TOCSY peaks but weak or missing intraresidue H_N -H_α NOEs, they also show intense interresidue $H_N(i)$-$H_\alpha(i-1)$ NOEs peaks. Besides the intensity of the NOE cross peaks, the H_α, H_N, and ^{15}N chemical shifts for these residues also show a downfield shift compared to those of random coil or α-helical residues. The relative downfield chemical shifts of these atoms in a β-strand conformation place the resonances in well-resolved regions in each of the three-dimensions, facilitating their assignment. The complete analysis of the NOESY spectrum led to the identification of three stretches of α-helices, namely, residue 8-18, 31-37, and 51-62, and of four stretches of β-strands, *i.e.*, residues 22-26, 41-45, 66-70, and 89-93 in the protein. Assignment of 86% the backbone amides was achieved based on the above analysis. Weak or no NOE connectivity was observed between residues 27-30, 74-78 and 81-86, which were therefore difficult to assign. The weak NOEs or absence of observable backbone amide protons are results of intermediate exchange (either conformational exchange or solvent exchange) broadening on the NMR time scale.

2.2.2.2. Assignment of the nonexchangeable resonances

Although the nonexchangeable protons can in principle be assigned from a ^{15}N-TOCSY-HSQC spectrum, the TOCSY transfer from the NH proton to β, γ, *etc.*, are often weak or absent for such an analysis. One of the main reasons for the decreased intensities is the dynamic range of water; as these experiments have to be recorded in at least 90% water sample to observe the amides, most magnetization is transferred to water. In addition, the increased solvent viscosity at low temperature results in slow protein correlations, which is responsible for some proton
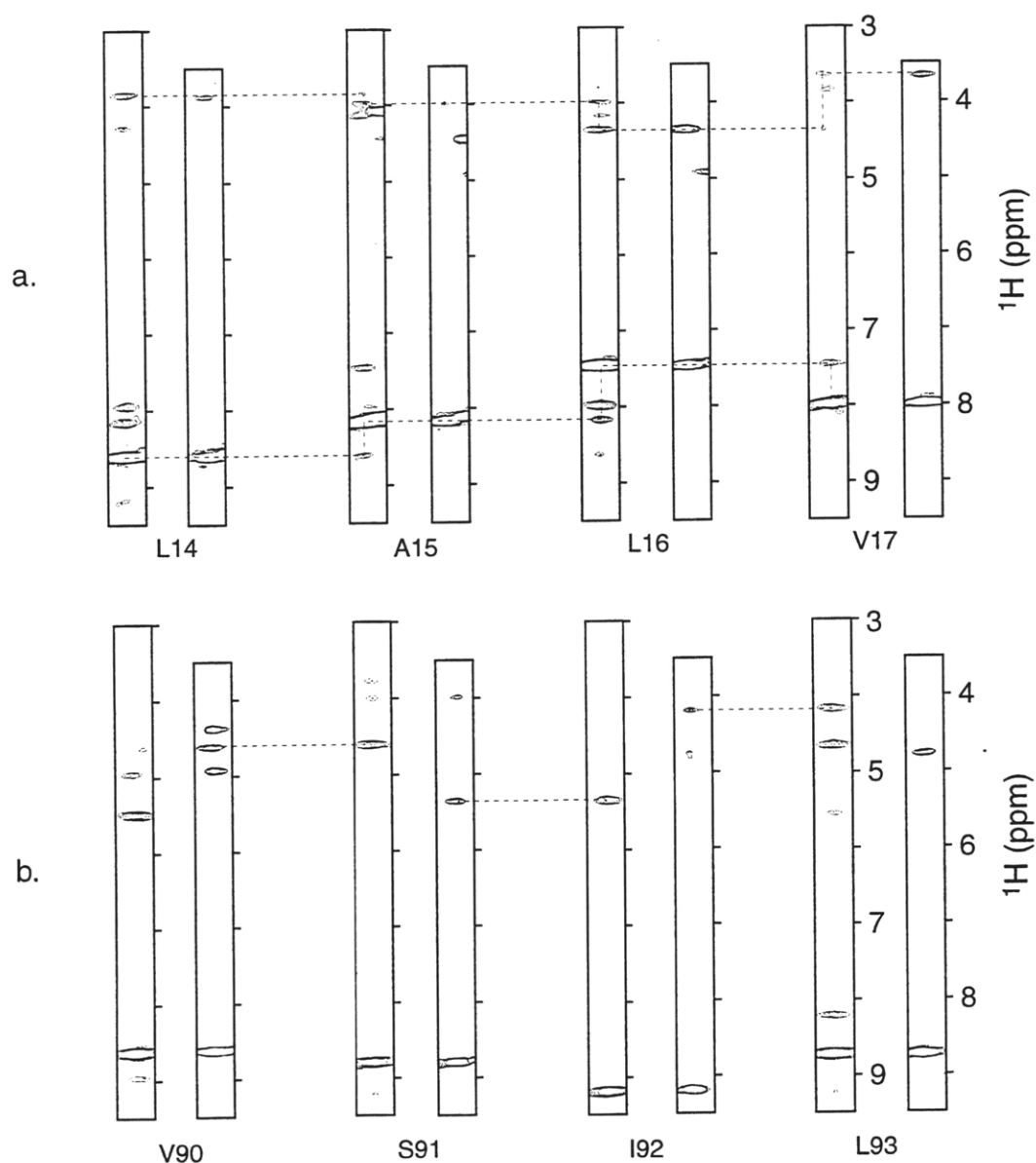
Figure 2.2.2: Selective representation of H_N, H_α regions in 3D ^{15}N NOESY-HSQC (longer slices) and ^{15}N TOCSY-HSQC (shorter slices) spectra of ^{15}N-labeled L30 protein. Alignment of NOESY and TOCSY spectra shows the cross peak intensities of four consecutive residues in **a**, α-helical, and **b**, β-sheet conformations. For a given residue, the correlation of H_N-H_α can be identified in a TOCSY-HSQC spectrum. By following the sequential NOEs in different slices, it is possible to identify the secondary structure adopted by a given residue.

linewidths larger than many of the proton-proton couplings. Since the ^{15}N-edited TOCSY-HSQC experiment relies heavily on the small ^{1}H-^{1}H scalar coupling constants to transfer magnetization, most relays beyond H_N-H_β correlation were absence, in the majority of cases preventing the correlation of H_N chemical shifts with those of side-chain protons (H_β and beyond). In the present case, most of the H_α protons and some H_β protons are assigned from the ^{15}N-TOCSY-HSQC spectrum. While it is impossible to increase the temperature for better sensitivity, a better way to observe the aliphatic protons would be to recorded experiments in 100% D_2O. A simple TOCSY (or PCOSY) spectrum in D_2O can help in assigning various aliphatic protons. In some cases where H_N-H_β correlation was not observed in the ^{15}N-edited TOCSY-HSQC spectra, the location of an H_β position was identified in a TOCSY or PCOSY spectrum acquired in D_2O through the H_α chemical shift assignment (provided that the H_α chemical shift was resolved). Concomitant with the assignments made in the TOCSY (or PCOSY) spectra, the H_β and H_γ chemical shifts can be corroborated with intraresidue H_N-H_β and/or H_N-H_γ NOE cross peaks in the 3D NOESY-HSQC spectrum. All Alanine (Ala) residues were easily assigned because of the relatively intense H_N -H_β correlation in the TOCSY-HSQC spectrum. A number of serine (Ser), threonine (Thr), valine (Val), isoleucine (Ile) and glycine (Gly) spin systems were identified on the basis of the characteristic coupling patterns in the homonuclear PCOSY and TOCSY spectra. Many aromatic residues were assigned based on the strong TOCSY relay peaks within ring protons, and characteristic NOEs between a ring H_δ group and H_N, H_α, H_β spin systems. Assignments of the three Arg were made through the backbone H_N and side chain H_ε NOESY-HSQC and TOCSY-HSQC correlation. A considerable degree of overlap for the H_β and H_γ resonances made this homonuclear based approach difficult for spin systems that extend beyond the β-methylene group, with lysines (Lys) and leucines (Leu) being the most troublesome cases.

In order to overcome problems associated with resonance overlaps and insufficient spin relays in the homonuclear-based side chain assignment, a ^{13}C/^{15}N double-labeled sample was prepared to for carbon-based assignment. The resolution of the side chain protons is improved by spreading the resonances into the ^{13}C dimension according to the chemical shift of the attached ^{13}C, (the ^{13}C of aliphatic side chains gives a wide chemical shift dispersion, from ~70 ppm to ~10 ppm). Besides helping in resolution enhancement, heteronuclear ^{13}C-based

experiments also help in increasing magnetization transfer. Contrary to the small three-bond homonuclear coupling constant (< 10 Hz), the large heteronuclear ^{13}C-^{13}C (~35 Hz) and ^{1}H-^{13}C (~120 Hz) one-bond couplings are known to transfer magnetization more efficiently, hence the through bond connectivity is improved.

The carbon HSQC spectrum can be differentiated into various regions containing different types of resonances. Among them, some resonances like the C_β of Leu, C_γ of Val , C_δ of Ile and Gly C_αs have distinct ^{13}C chemical shifts (Wishart *et al.*, 1995) and can be easily identified. A more helpful experiment would be to record a constant time CT-HSQC experiment. In the CT-HSQC, depending on the constant time period, the cross peaks can have different signs. When T = 1 / J_{CC} the sign of the ^{13}C magnetization is opposite for carbons coupled to an odd, relative to an even number, of other aliphatic carbons. Figure 2.2.3 illustrates this point for the L30 protein. If, on the other hand, T = 2 / J_{CC}, all cross peaks will have the same sign. An additional advantage is that some spin system identifications can be made simply by observation of the aliphatic ^{13}C chemical shifts as well as the sign of cross peaks in the ^{13}C-edited CT-HSQC spectra even prior to conducting a more elaborate specific assignment. The above information is particularly helpful in assigning the following spins: ^{13}C$_\delta$s of Iles have the most upfield chemical shifts (< 17 ppm), while ^{13}C$_\beta$s of Thr have the most down field chemical shift (~70 ppm), but opposite sign to the ^{13}C$_\alpha$s that are close by. Within the ^{1}H$_\alpha$-^{13}C$_\alpha$ region, ^{13}C$_\beta$ of Ser can be differentiated from other ^{13}C$_\alpha$s by the presence of two ^{1}H$_\beta$ cross peaks at the 60 ppm region, and ^{13}C$_\alpha$s of Gly occupy the most upfield region (~ 42 ppm) with a set of two H$_\alpha$s. All seven Thr ^{1}H$_\beta$s, nine sets of Gly ^{1}H$_\alpha$s and eight of ten Ser ^{1}H$_\beta$s were identified in the CT-HSQC spectra. In addition, five Ile ^{1}H$_\delta$s were identified unambiguously from the chemical shifts of ^{13}C$_\delta$s, two others were not ascertained because of the complexity introduced by some nearby ^{13}C$_{\gamma2}$ chemical shifts.

Although the categorization of spins can be made in the HSQC spectrum, the assignment of most spins to specific amino acids in the sequence has not yet become clear, except for a few ^{1}Hs with distinct chemical shifts (*e.g.*, 0.13 ppm of Ile18 ^{1}H$_\delta$s). A more extensive side chain assignment using a ^{13}C edited HCCH-TOCSY (Clore *et al.*, 1990) (^{1}H-^{13}C-^{13}C-^{1}H total

correlation spectroscopy) experiment acquired on the MIT Bitter Lab 500 MHz instrument. In this experiment, the ^{1}H carrier was positioned at 2.9 ppm to be in between the aliphatic side chains, while ^{13}C carrier was positioned at 40 ppm between ^{13}C$_\alpha$ and side chain ^{13}Cs. The delay for the ^{1}H-^{13}C transfer was set to 1.5 ms, ~$1/4J_{CH}$, and that for the ^{13}C-^{13}C transfer was set to 3.25 ms, about $1/8J_{CC}$. The ^{13}C-^{13}C isotropic mixing was accomplished with a 24 ms DIPSI-3 (Shaka *et al.*, 1988) applied along the y-axis with a 7 kHz bandwidth. No carbonyl decoupling was applied during evolution, and a GARP (Shaka *et al.*, 1985) decoupling for carbons was applied during acquisition.

In addition to the HCCH-TOCSY experiment, a ^{13}C-edited NOESY-HSQC spectrum (Clore *et al.*, 1990) was also recorded on a Varian Inova 600 MHz with 100 ms mixing time. Besides the contributing intraresidue NOE information for assisting the HCCH-TOCSY side chain assignment, the ^{13}C-edited NOESY-HSQC spectrum also provided the critical information of the approximate long-range NOEs that would help to define a full high-resolution three-dimensional fold for the protein. In this experiment, the ^{1}H carrier was placed at 2.62 ppm and ^{13}C at 43.64 ppm, similar to those in the HCCH-TOCSY experiment. While a GARP decoupling sequence was applied during acquisition, an additional carbonyl decoupling was implemented during the evolution period, using a shaped 180° SEDUCE-1 (McCoy & Mueller, 1992) decoupling sequence.

Detailed assignments of the side chain ^{1}H and ^{13}C resonances of L30 protein were made using the ^{13}C-edited HCCH-TOCSY spectrum in conjunction with strong intraresidue NOEs identified in the ^{13}C-edited NOESY-HSQC spectrum, and the ^{1}H$_\alpha$ assignments made in the ^{15}N TOCSY-HSQC experiment. Similar to the homonuclear experiment, the most easily identified spins were also Gly, Ala, Thr, Val and Ser residues but with less ambiguous identification in the HCCH-TOCSY spectrum as described below. For glycines, two H$_\alpha$s are attached to the same C$_\alpha$, so a symmetric pattern appears about the diagonal. This characteristic pattern is also seen for β-methylene protons. The methylene H$_\beta$s not only have symmetric patterns about the diagonal but they are also associated with cross peaks to the H$_\alpha$ protons. For serines, the β-methylene protons are distinct as they resonate more downfield compared to others, such as Tyr, Phe, Asn and Asp
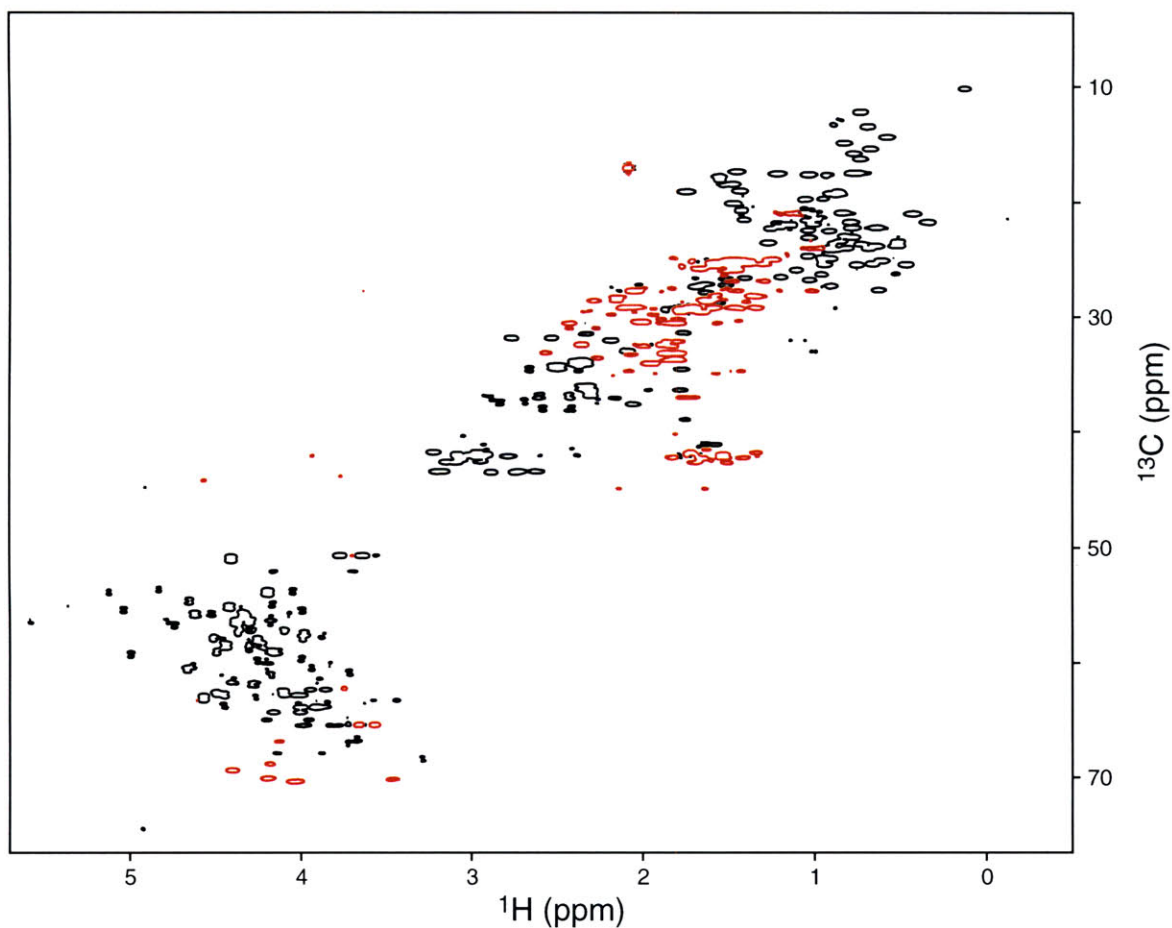
Figure 2.2.3: A ^{1}H-^{13}C CT-HSQC spectrum of the L30 protein acquired with T = 27 ms at 10˚C. The number of aliphatic carbons attached to a given ^{13}C nucleus determine the sign of the ^{13}C magnetization, in which odd-number coupled aliphatic carbons (shown in black) have opposite sign relative to even-number coupled aliphatic carbons (shown in red). Since the chemical shifts of carbonyl ^{13}C and aromatic ^{13}C are further away from the rest of ^{13}C nuclei, it is not sufficiently decoupled during the evolution period, thus these nuclei do not produce any effect on the neighboring ^{13}C nuclei. In addition, the intensities of the observed cross peaks are attenuated by the ^{13}C transverse relaxation; hence, some resonances (*e.g.* Glys) are missing in the spectrum.

of other AMX spin systems. Using this approach, all of the nine Glys H_αs were assigned in association with the correlations made in the ^{15}N-edited TOCSY-HSQC, and ten sets of Sers H_βs were identified and connected to the backbone exchangeable protons via their own H_αs.

The assignment of Thr and Val spin systems were based on the characteristic connectivity originating from the H_α to H_β and H_γ methyl protons. Differentiation of the two types of spin systems were made on the basis of different $^{13}C_\beta$ chemical shifts. The Thr $^{13}C_\beta$ chemical shifts are at 70-73 ppm, while those of Val are at 31-37 ppm. In addition, there is only one $^{13}C_\gamma$ methyl in each Thr residue, while there are two $^{13}C_\gamma$ methyls (mostly with different chemical shifts) in each Val residue. The two methyl groups in the Val residues usually display strong intraresidue NOEs in the ^{13}C-edited NOESY-HSQC spectrum. Excluding residues in the flexible region (Thr30, Val81 and Val87) where only tentative assignments were made, all of the remaining six Thr and seven Val were unambiguously identified.

A complete Ile spin systems identification was made starting from H_δ, since the directly-attached $^{13}C_\delta$ has the most upfield chemical shifts and fall in a distinct region in the ^{1}H-^{13}C HSQC spectrum. H_δs can also be distinguished from other methyl groups in 2D-homonuclear COSY or short mixing time TOCSY spectra, since they often relay to two methylene protons, while others relay to only one proton. Ile $H_{\gamma2}$ methyls were identified from the characteristic $^{13}C_{\gamma2}$ chemical shifts around 17 ppm. These were easily differentiated from the nearby $^1H_\beta$ /$^{13}C_\beta$s of Ala through both characteristic intraresidue NOEs and HCCH-TOCSY correlation patterns. HCCH-TOCSY was further used to identify $H_{\gamma1}$s and H_βs by the relays from the δ methyl group.

A similar approach was also used for the leucines as they contain two δ methyl groups and one γ proton. Precaution was needed with this approach, since without further relays, the same type of correlation pattern also occurs in Val residues (two H_γ and one H_β protons). The ambiguities were resolved by confirming the relays to the Leu H_β resonances. The Leu C_β also has a characteristic chemical shift in the ~43 ppm region, and was easily identified by the distinct signs in the CT-HSQC spectrum (T = $1/J_{CC}$).

The most difficult assignments were again the twelve lysine (Lys) residues in L30 protein. The lack of chemical shift resolution and reduction of effective magnetization transfer through long side chains rendered the HCCH-TOCSY experiment less useful in distinguishing different lysine residues. Therefore, other than the H_β protons that were assigned with confidence, the rest of the spins only have tentative assignments.

2.2.2.3. Standard homonuclear experiment for specific assignments

In addition to the heteronuclear experiments which provide majority of the resonance assignments and NOE distance information, 2D NMR spectra, including NOESY, PCOSY (Marion & Bax, 1988), and TOCSY experiments, are necessary for assisting in resonance identification. These experiments, in particular, PCOSY and TOCSY with different mixing time acquired in D_2O, assisted the spin correlation assignments from H_α proton to side chain protons as described above. Short mixing time (11 ms) TOCSY experiment, in conjunction with short mixing time (50 ms) NOESY spectrum assisted in the stereo-specific assignment of many H_β protons. In addition, the aromatic protons of six tyrosines (Tyr) and two phenylalanines (Phe) were primarily assigned from TOCSY and NOESY spectra. The complete intraresidue aromatic protons were identified in a TOCSY relayed spectrum (35 ms) and were further connected to their H_β-methylene and H_α protons from a short mixing time NOESY spectrum.

2.2.2.4. Triple resonance experiments

Since the first description of triple-resonance based assignments as shown for calmodulin (Ikura *et al.*, 1990), 3D triple-resonance experiments have offered an alternative approach to classical NOE-based strategies for sequential assignment. In contrast to observation of characteristic short-range NOEs, these experiments correlate backbone ^1H_N, ^{15}N, $^1H_\alpha$, $^{13}C'$ (carbonyl carbon), and side-chain $^1H_\beta$ and $^{13}C_\beta$ spins using one-bond and two-bond scalar coupling interactions. These are particularly useful because these bond couplings, unlike NOEs, are independent of the structure. Thus, triple resonance experiments in practice offer less ambiguous sequential assignments than the classical NOE strategy. But these experiments

require a high sample concentration and rigorous optimization. For the L30 protein, though an HNCA experiment was tried on a 0.7 mM $^{13}C/^{15}N$-labeled L30 protein, sufficient signal was not observed. Also, the protein started aggregating during the course of the experiment. No further triple-resonance experiments were tested on this sample as a result of this gradual aggregation.

Overall, with the combination of both 2D homonuclear and 3D heteronuclear experiments, approximately 80% of the ^{1}H, ^{15}N and ^{13}C chemical shift assignments of L30 protein in the free form were made. These chemical shifts are tabulated in Table 2.1. The general acquisition parameters used in all the homonuclear and heteronuclear experiments performed on the free L30 protein are listed in Table 2.2.

The chemical shifts of the various assigned resonances were further analyzed to determine the secondary structure of the protein. As mentioned earlier, the chemical shifts of all amino acids are directly related to the adopted secondary structures and the surrounding environment. In particular, the $^{1}H_{N}$, $^{1}H_{\alpha}$, ^{15}N, $^{13}C_{\alpha}$ and $^{13}C'$ values are directly related to the backbone ϕ, ψ dihedral angles, which can be used as reliable indicators for secondary structure alignment. With the assignments in hand (except for $^{13}C'$), it is possible to calculate chemical shift deviations of $^{1}H_{N}$, $^{1}H_{\alpha}$, ^{15}N and $^{13}C_{\alpha}$ from the corresponding average random coil values and use this to map out the conformational propensities of the backbone. According to the empirical chemical shift index, the average secondary shifts of ^{15}N, $^{1}H_{N}$, $^{13}C_{\alpha}$, and $^{1}H_{\alpha}$ of residues in α-helices are -1.7 ± 0.3, -0.19 ± 0.1, 2.6 ± 0.5 and -0.38 ± 0.1 ppm, respectively, whereas in β-sheets these are 1.2 ± 0.3, 0.29 ± 0.1, -1.4 ± 0.5 and 0.38 ± 0.1 ppm, respectively (Wishart & Sykes, 1994). Figure 2.2.4 shows the summary of ^{15}N, $^{1}H_{N}$, $^{13}C_{\alpha}$, and $^{1}H_{\alpha}$ secondary shifts plotted for each residue. Four stretches of α-helices (with one incompletely identified) and four stretches of β-sheets can be identified in L30 protein in the free form, which is consistent with the NOE observation. It is notable that the largest secondary shifts are in regions of the middle of helices or sheets. The more reliable observation comes from the $^{13}C_{\alpha}$ and $^{1}H_{\alpha}$ secondary shifts. ^{15}N and $^{1}H_{N}$ values on the other hand show more deviations, because they are not only affected by the backbone dihedral angle but also influenced by the strength of any hydrogen bonds (Wishart & Sykes, 1994).

Table 2.1: ^{1}H, ^{15}N and ^{13}C chemical shift list of free form L30 protein at 10°C. Proton chemical shifts are referenced to internal TSP. Nitrogen and carbon chemical shifts are referenced to external NH_4Cl and TSP, respectively. The errors in chemical shifts are ± 0.02, 0.2, 0.3 ppm for proton, nitrogen and carbon, respectively. Tentative assignments are indicated in ().

Residue	H$_N$/N	Hα/Cα	Hβ/Cβ	Hγ/Cγ	Hδ/Cδ	Hε/Cε	εNH2/εN
A2		4.42/51.0	1.55/18.1				
P3		4.58/62.9	1.89,2.36/23.3	2.05/27.7	3.76,3.65/50.6		
V4	8.50/121.4	4.08/62.5	2.06	32.90	1.00/21.5		
K5	8.60/125.6	4.39/56.4	1.78,1.71/33.1	1.52,1.45	1.85,1.97	3.02	
S6	8.48/117.2	4.44/58.4	4.01/62.7				
Q7	8.74/122.7	4.31/56.4	2.12,2.05/28.6	2.35/33.9			7.64 6.53/112.0
E8	8.45/121.1	4.25/57.0	2.44/36.46	2.44 2.60/36.9			
S9	8.44/116.5	4.47/58.9	4.20 3.97/63.73				
I10	8.59/122.0	3.83/63.4	2.05/37.57	1.02,1.34,1.46/17.6,29.2	0.68/13.4		
N11	8.41/120.3	4.27/57.0	2.89/37.42				7.59,7.01/109.2
Q12	8.09/120.2	4.17/58.9	2.16,2.28/29.2	2.48/34.29			7.64,6.93/112.0
K13	7.93/119.5	4.16/59.7	1.62,1.83	1.28	1.67	2.92	
L14	8.60/119.5	3.85/57.6	1.34,1.73/41.8	1.50/27.30	0.60,0.50/25.1,23.8		
A15	8.16/121.2	3.98/55.5	1.55/17.7				
L16	7.44/116.9	4.36/57.0	1.59 1.81/40.1	1.64/27.17	1.02,0.91/24.8,24.2		
V17	7.98/124.5	3.66/66.5	2.33/31.4	1.25,1.00/23.5,20.0			
I18	7.98/118.7	3.70/62.8	1.98/36.3	0.76,1.02,1.23/17.5,27.8	0.13/10.16		
K19	7.65/117.6	4.30/58.6	1.97,1.73	1.50	1.72	2.99	
S20	8.05/110.1	4.99/59.1	3.87,4.12/67.7				
G21	9.07/115.2	4.57,4.77/44.0					
K22	9.10/129.1	5.03/55.4	1.77,1.74	1.53,1.38			
Y23	8.72/122.5	5.56/56.3	2.93,2.83/41.2		6.67/133.4	6.92/118.7	
T24	9.21/112.6	4.67/60.4	4.05/70.1	1.05/19.7			
L25	8.46/122.0	5.11/53.9	2.14,1.62/44.9	2.03/27.1	1.19,1.10/26.5,25.9		
G26	8.35/107.1	4.68,4.44/44.7					
Y27	8.60/123.5	3.90/63.3	3.31,3.19/39.3		7.02/133.2	6.88/119.3	
K28	8.95/119.8	3.70/60.7	1.85,2.00/32.5	1.69			
S29	9.28/115.0	4.15/60.9	3.95,3.88/62.1				
T30	8.02/120.7	3.77/67.9	4.12/66.6	(1.02/22.0)			
V31	8.79/124.0	3.28/68.2	1.75/31.35	0.64,0.42/23.8,21			
K32	7.39/119.3	3.91/61.4	1.80,1.86	1.46,1.66		2.92	
S33	7.81/114.0	4.24/61.7	4.00,3.62/63.3				
L34	7.85/123.9	4.34/54.2	1.73,1.64/41.5	1.62/27.9	0.93,0.88/27.8,23.5		
R35	8.53/120.9	3.98/59.5	1.91,1.80	1.57,1.34	2.73,2.88		6.98/84.0
Q36	7.93/113.5	4.29/56.0	2.35,2.07/29.5	2.63,2.36/34.5			7.51,6.87/114.5
G37	7.83/108.6	4.09,3.98/46.8					
K38	8.07/115.7	4.34/55.8	1.92,1.64	1.52	2.99		
S39	7.41/111.9	4.12/62.2	3.44,3.58/63.0				
K40	9.30/124.1	4.50/55.7	2.10,1.46	1.53			
L41	7.67/120.7	4.61/54.8	1.17,1.82/45.3	1.28/29.2	0.60,0.80/27.7,24.0		

I42	8.50/119.3	5.50/58.5	1.62/41.3	0.87,1.00/19.3,28.1	0.77/15.8		
I43	8.96/123.2	5.27/59.5	1.64/41.2	0.87,1.02/19.1,27.7	0.67/15.4		
I44	8.54/124.2	4.64/60.2	1.53/41.1	1.21,0.82/19.4,27.8	0.82/14.9		
A45	8.72/129.1	4.67/53.4	1.72/18.5				
A46	8.83/123.6	4.33/55.3	1.44/19.1				
N47	8.28/111.7	4.82/52.4	3.19,2.94/36.5				7.76,7.28/112.1
T48	7.60/120.5	4.50/62.2	4.02/70.1	1.52/19.1			
P49		4.47/63.69	2.57/33.1	2.16,1.89/28.3	3.69 4.16/52.0		
V50	8.67/123.7	3.71/66.9	2.19/32.0	1.16,1.05/22.0,20.9			
L51	8.88/120.6	4.21/58.4	1.53,1.71/41.9	1.68/27.5	0.93,0.90/24.9,25.2		
R52	6.93/116.6	4.28/57.0	1.57,1.91	1.50,1.40	2.62,2.73		7.06/83.8
K53	8.52/119.9	3.87/61.4	2.07,1.74	1.50			
S54	8.62/112.2	4.41/57.9	4.20,4.01/64.1				
E55	7.69/123.8	4.22/59.7	2.02/30.4	2.30/36.7			
L56	8.36/118.4	4.00/58.6	1.33,1.85/42.0	1.69/26.7	1.02,1.02/26.8,24.9		
E57	8.68/116.6	3.80/60.0	2.03,2.35/30.5	2.70,2.28/37.4			
Y58	8.18/122.1	4.38/57.2	3.19,3.11/37.9		6.54	6.58	
Y59	8.26/116.3	4.11/62.4	3.11,2.84/38.8		7.27	7.03	
A60	9.00/124.3	4.06/57.0	1.44/17.35				
M61	8.21/119.9	4.20/58.8	2.26,2.44/31.9	2.77,2.53/31.0		2.09/17.0	
L62	7.94/112.0	3.98/57.7	1.71,1.51/42.6	1.40/26.7	0.77,0.64/23.0,25.4		
S63	7.57/109.8	4.50/57.8	3.96,4.19/64.8				
K64	7.88/120.6	4.00/57.3	2.09,1.92	1.78,1.43		3.06	
T65	8.84/118.2	4.26/62.8	3.46/70.0	1.05/21.6			
K66	8.18/127.5	4.08/57.0	1.85,1.80	1.57,1.48			
V67	8.11/121.3	4.47/60.7	1.78/34.5	0.32,0.77/21.8,21.7			
Y68	9.12/128.1	4.72/56.6	2.80,2.85/41.1		7.09	6.90	
Y69	8.09/127.2	4.60/55.7	2.53,2.93/36.9		7.05	6.70	
F70	8.36/126.4	4.50/57.4	3.03,2.84/41.1		7.26	7.26	
Q71	9.01/128.0	4.15/56.1	1.83,1.98/27.3	2.35,2.40/34.1			7.43,6.96/113.0
G72	7.08/107.2	3.77,4.18/44.6					
G73	8.46/105.9	4.47,4.17/44.7					
N74	9.38/118.8	4.28	2.90/37.1				7.92,7.09/111.2
N75	9.11/123.2						7.88,6.90/114.3
E76	8.04/121.9	4.16/60.1	2.14,2.30	2.44,2.57/38.0			
L77	9.11/123.2	3.62/57.2	1.33,1.82/42.0	1.05/33.0	0.50,0.14/26.3,21.4		
G78	7.65/104.8	4.04,3.43/47.7					
T79	7.99/117.6	4.08/66.2	4.19/68.6	1.25/22.3			
A80	8.15/125.2	4.15/55.0	1.46/18.4				
V81	7.35/106.4	4.31/	(2.35/30.7)	(0.64,0.90/22.2,19.6)			
G82	7.79/107.9	3.92,3.78/45.4					
K83	8.18/117.3	4.17/56.4	1.90				
L84	8.40/120.2	4.30	(1.65,1.52/40.1)	(2.36)	(0.82,0.93/24.1,24.4)		
F85	6.48/114.6	4.95/60.7	3.36 3.08/40.9		7.21	7.34	
R86	8.40/117.3	4.39/61.3	1.73	(1.41)	3.19		7.35/85.7
V87	8.00/121.4	4.50/62.5	(1.16/38.9)	(0.41,0.54/22.6,23.8)			

G88	9.17/113.2	3.92,3.78/46.3		
V89	7.20/116.0	5.51/59.0	2.21/33.7	1.02,0.85/23.1,18.5	
V90	8.62/125.2	4.65/60.1	1.77/36.3	0.77,0.83/22.3,20.9	
S91	8.79/119.6	5.35/54.9	3.98,3.76/65.3		
I92	9.18/126.0	4.18/60.5	2.16/37.1	0.73,1.00,1.34/17.5,29.6	0.73/12.2
L93	8.72/129.1	4.75/56.5	1.35 1.99/43.0	1.55/28.8	0.68,0.74/24.0,25.5
E94	8.21/116.2	4.59/57.9	2.28,1.80/33.6	2.30,2.35/36.1	
A95	9.27/124.8	4.02/53.8	1.46/20.1		
G96	8.51/105.7	3.72,3.69/46.5			
D97	8.83/124.5	4.63/54.6	2.90,2.41/41.3		
S98	7.72/112.1	4.44/61.7	3.97,3.79/65.3		
D99	8.45/126.3	4.81/53.7	3.04,2.60/40.1		
I100	7.89/119.3	3.99/55.8	1.73/38.9	0.73,1.25,1.48/16.2 26.8	0.57/14.4
L101	8.56/116.6	4.16/56.1	1.83,1.66/42.19	1.41	0.96,0.71/25.5,23.1
T102	7.67/108.0	4.50/62.5	4.40/69.1	1.18/22.1	
T103	7.46/110.8	4.44/62.6	4.20/69.9	1.14/21.0	
L104	7.61/122.1	4.41/55.2	1.54 1.41/42.2	1.50/26.7	0.46,0.50/25.5,23.5
A105	7.81/129.2	4.18/53.9	1.40/20.7		

Table 2.2: Data acquisition parameters and pulse sequences used in free form L30 protein NMR experiments recorded at 10°C. The four subdivisions in each NMR dimension (*F1*, *F2*, or *F3*) are: **a**, complex data points, **b**, spectral width (Hz), **c**, carrier position (ppm) and **d**, nuclei. In a 2D experiment, *F2* is the direct acquisition dimension, whereas *F1* is the indirect detection dimension. In a 3D experiment, *F3* is the direct acquisition dimension, whereas *F1* and F2 are the indirect detection dimensions.

Molecule	Experiment	*F3*(a, b, c, d)	*F2*(a, b, c d)	*F1*(a, b, c, d)	Mixing time (ms)	Reference
Nonlabeled L32 Protein	NOESY (Varain 600)	-	2048, 6999.7, 4.91. ^{1}H	512, 6999.7, 4.91, ^{1}H	50, 100, 150, 200	
	NOESY-watergate (Varain 600)	-	2048, 14000, 4.91, ^{1}H	512, 14000, 4.91, ^{1}H	100, 200	
	dq-COSY (Varain 600)	-	2048, 6999.7, 4.91, ^{1}H	512, 6999.7, 4.91, ^{1}H	-	(Rance *et al.*, 1983) (Marion & Wüthrich, 1983)
	P-COSY (Bitter 501)	-	2048, 5500, 4.91, ^{1}H	512, 5500, 4.91, ^{1}H	-	(Marion & A.Bax, 1988)
	TOCSY (Varain 600)	-	2048, 6999.7, 4.91, ^{1}H	450, 6999.7, 4.91, ^{1}H	35, 50	(Brauschweiler & Ernst, 1983)
	TOCSY (Bitter 501)	-	2048, 60002.4, 4.91, ^{1}H	512, 6002.4, 4.91, ^{1}H	11	
^{15}N Labeled L32 Protein	FHSQC(Varain 600)	-	1024, 7000, 4.91, ^{1}H	128, 2000.0, 118.41, ^{15}N	-	(Mori *et al.*, 1995)
	T1ro, T1, NOE Relaxation (Varain 600)	-	512, 7000, 4.91, ^{1}H	128, 2000.0, 118.41, ^{15}N	-	(Dayie & Wagner, 1994)
	^{15}N Watergate NOESY-	512, 6000.2, 4.91, ^{1}H	128, 6002.4, 4.91, ^{1}H	32, 1600, 116.5, ^{15}N	100	(Mori *et al.*, 1995)

^{15}N, ^{13}C Labeled L32 Protein	^{15}N TOCSY-HSQC-watergate (Varian 600)	512, 6999.7, 4.91, ^{1}H	128, 7000.0, 4.91, ^{1}H	32, 2000, 118.41, ^{15}N	50	(Marion et al., 1989)
	^{13}C HSQC-CT (Varian 600)	-	1024, 7000, 4.91, ^{1}H	105, 3850, 43.64, ^{13}C	-	(Santoro & King, 1992)
	^{13}C HSQC-CT (Varian 600)	-	1024, 7000, 4.91, ^{1}H	512, 10999.6, 39.99, ^{13}C	-	(Santoro & King, 1992)
	^{13}C FHSQC (Varian 600)	-	1024, 7000, 4.91, ^{1}H	512, 10999.6, 39.99, ^{13}C	-	(Mori et al., 1995)
	^{13}C HSQC-NOESY (Varian 600)	512, 6999.7, 2.62, ^{1}H	128, 4000, 2.62, ^{1}H	32, 3850, 43.64, ^{13}C	100	(Clore et al., 1990)
	^{13}C HCCH-DIPSI (Bitter 501)	512, 6000.2, 2.90, ^{1}H	128, 6000.2, 2.90, ^{1}H	32, 3210, 40.0, ^{13}C	12	(Clore et al., 1990)
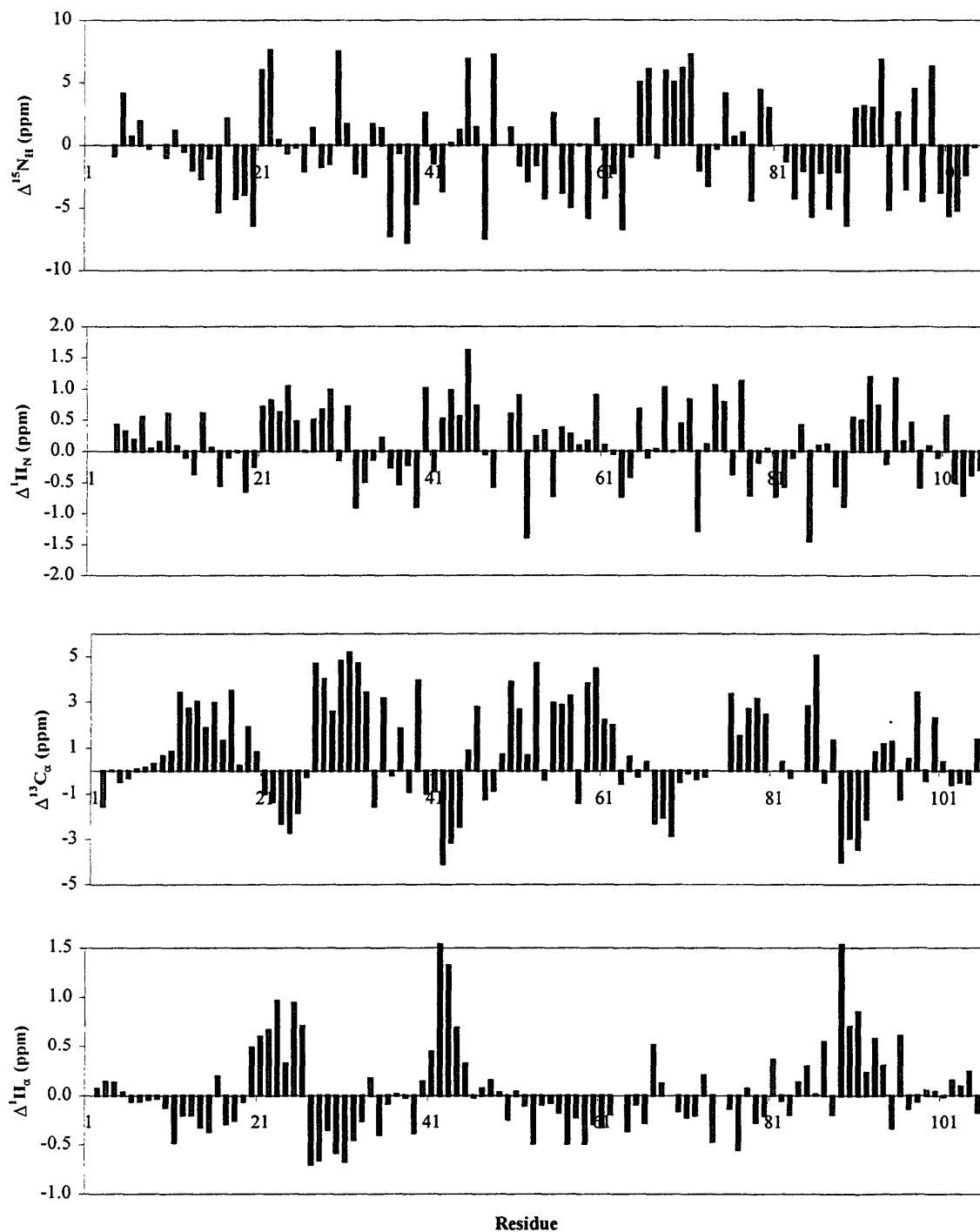
Figure 2.2.4: Summary of secondary ^{15}N, $^{1}H_N$, $^{13}C_\alpha$ and $^{1}H_\alpha$ chemical shifts for the L30 protein in the free form. The deviations of the chemical shifts are derived from comparisons to the chemical shift index (CSI) values reported by Wishart *et. al.*, 1994. Residues with chemical shift deviation absolute values greater than 0.1 or 0.7 ppm for respective H_α or C_α are particularly useful in identification of protein secondary structure.

2.2.2.5. Stereospecific assignments

Stereospecific assignment is a necessary step in determining the side chain torsion angle for determination of a high-resolution structure in NMR. In order to determine the torsion angle χ_1 about the C_α-C_β bond, stereospecific assignment of atoms associated with the C_β prochiral center of many of the amino acids was necessary. There are two types of prochiral atoms at C_β positions, those with two β-methylene protons, and others with two γ-carbons. Stereospecific assignment of prochiral centers was primarily based on measuring $^3J_{H\alpha\text{-}H\beta}$ coupling constant, in combination with typical NOE patterns associated with the adopted conformation.

In the case of two β-methylene protons, if the two measured H_α-H_β coupling constants are < 5 Hz, the corresponding to $\chi_1 = +60°$; whereas if one has a large coupling constant (> 10 Hz) and one has a small coupling constant (< 5 Hz), $\chi_1 = -60°$ or $\chi_1 = +180°$. Additional information about the relative intensities of the H_N-H_β and H_α-H_β NOEs is needed to distinguish between $\chi_1 = -60°$ and $\chi_1 = +180°$ (Wagner *et al.*, 1987) (see **a** in Figure 2.2.5). Pairs of H_α-H_β coupling constants that differ by less than 5 Hz usually indicate side-chain averaging about χ_1. H_α-H_β coupling constants for residues with two β-protons can be obtained from a DQF-COSY experiment, or a short mixing time (11 ms) TOCSY experiment (Cai *et al.*, 1995). Because the buildup rate of a TOCSY peak depends on the size of the scalar coupling constant, qualitative analysis of cross peak intensities therefore can be used to estimate coupling constants (Clore *et al.*, 1991). From a DQF-COSY spectrum, the quantitative measurements can be made for the large coupling constants (> 5 Hz) but it is difficult for the small coupling constants (< 5 Hz). The absence of cross peaks in this spectrum may not be referred to small couplings as there may be other reasons why cross peaks are missing; nevertheless, this is the only method available for large protein. Overall, excluding residues with resonance that could not be identified because of severe overlap, a total of 40 residues with β-methylene protons were stereospecifically assigned.

For residues with two γ-groups (*i.e.*, Thr, Ile and Val), there is only one H_α-H_β coupling constant. In these cases if the measured $J_{\alpha\beta} > 10$ Hz, this correspond to $\chi_1 = -60°$, whereas if $J_{\alpha\beta}$

a

	60	180	-60
$^3J_{\alpha\beta2}$	w	w	s
$^3J_{\alpha\beta3}$	w	s	w
Hα-Hβ2 NOE	s	s	w
Hα-Hβ3 NOE	s	w	s
HN-Hβ2 NOE	w	s	w
HN-Hβ3 NOE	s	s	w

b

	180	-60	60
$^3J_{\alpha\beta}$	s	w	w
Hα-Hγ1 NOE	s	s	w
Hα-Hγ2 NOE	s	w	s
HN-Hγ1 NOE	w	s	w
HN-Hγ2 NOE	s	s	w

Figure 2.2.5: Scheme for obtaining stereospecific assignments of **a**, β-methylene protons and **b**, γ-methyl protons on the basis of a qualitative analysis of the relative intensities of cross peaks in 3D ^{15}N NOESY-HSQC, 3D ^{13}C NOESY-HSQC,^{15}N TOCSY-HSQC and 11 ms ^{1}H TOCSY experiments. The expected patterns of cross-peak intensities are indicated for the three prefered χ_1 rotamers. A strong cross peak is indicated by the letter s, and a weak or missing peak is indicated by the letter w. The β and γ positions are defined according to the IUPAC-IUB convention.

< 5 Hz corresponds to either $\chi_1 = +60°$ or $\chi_1 = +180°$. For Thr, Ile and Val residues, which contain only one β-proton, the H_α-H_β coupling constants were measured from the antiphase splitting of the H_α-H_β cross peaks in the DQF-COSY spectrum collected in D_2O after incorporation of linewidth corrections. Stereospecific assignments for the methyl groups of the valine residues were obtained on the basis of the relative intensity of the intraresidue H_N-H_γ and H_α-H_γ NOEs (Zuiderweg *et al.*, 1985) (see **b** in Figure 2.2.5).

2.2.3. Backbone relaxation studies

Recently, relaxation measurements have gained recognition as an important tool for studying the dynamic properties of macromolecules. Preliminary studies of the backbone dynamic properties of the free form L30 protein were carried out using heteronuclear NOE, spin-lattice (T_1) and spin-spin (T_2) ^{15}N relaxation measurements. Since the T_1 values and heteronuclear NOEs are sensitive to high frequency motions (10^8-10^{12} s^{-1}) and T_2 values are a function of much slower processes, it is possible to explore dynamic events occurring over a large range of time scale (micro- to millisecond time scales). In addition to determining the overall correlation properties of the L30 protein in the absence and presence of RNA on the basis of the T_1 and T_2 relaxation data, these measurements are also helpful in understanding the local motional properties of the protein that might be important for RNA recognition.

The theoretical description for the T_1, T_2 relaxation rates and the NOE enhancement has been described in detailed in the literature (Abragam, 1961). In summary, they can be described by the following equations:

$$1 / T_1 = d^2 \ [J(\omega_A - \omega_X) + 3J(\omega_X) + 6J(\omega_A + \omega_X)] + c^2 \ J(\omega_X) \tag{2.3}$$

$$1 / T_2 = 0.5 d^2 \ [4 \ J(0) + J(\omega_A - \omega_X) + 3J(\omega_X) + 6J(\omega_A) + 6J(\omega_A + \omega_X)] + \tfrac{1}{6}c^2 \ [3J(\omega_X) + 4J(0)]$$

$$(2.4)$$

$$NOE = 1 + \{(\gamma_A / \gamma_X) \ d^2 \ [6J(\omega_A + \omega_X) - J(\omega_A - \omega_X)] \ T_1\} \qquad (2.5)$$

with $d^2 = 0.1\gamma_A^2\gamma_X^2 \ h^2 / (4\pi^2)(1 / r^3_{AX})^2$ and $c^2 = (2/15) \ \gamma_X^2 \ H_0^2(\sigma_\parallel - \sigma_\perp)^2$. $A = {}^1H$, $X = {}^{15}N$, γ_i is the gyromagnetic ratio of spin i, h is Planck's constant, r_{AX} is the internuclear ${}^1H$-${}^{15}N$ distance, H_0 is the magnetic field strength, $\sigma_\parallel$ and $\sigma_\perp$ are the parallel and perpendicular components of the axially symmetric ${}^{15}N$ chemical shift tensor, and $J(\omega_i)$ is the spectral density function. The assumption of an axially symmetric chemical shift tensor has been shown to be valid for peptide bonds, with $\sigma_\parallel - \sigma_\perp = -160$ ppm (Hiyama *et al.*, 1988).

The spectral density function $J(\omega_i)$ of a protein in solution depends on both the overall motion of the macromolecule as a whole and on the internal motions of the ${}^1H$-${}^{15}N$ bond vector (Lipari & Szabo, 1982), which can be described as:

$$J(\omega) = S^2 \ \tau_c / [1 + (\omega\tau_c)^2] + (1 - S^2) \ \tau / [1 + (\omega\tau)^2] \qquad (2.6)$$

where S^2 is a generalized order parameter, measuring the degree of spatial restriction of internal motions of the N-H bond vector, τ_c is the effective correlation time for the overall motion, and $1/\tau = 1/\tau_c + 1/\tau_e$, where τ_e is an effective correlation time describing the rapid internal motion. Assuming that $[(\omega_A \pm \omega_X)\tau_e]^2 \ll 1$, and $(\omega_X\tau_m)^2 \gg 1$, the T_1, T_2 and NOE relaxation terms in equation 2.3 - 2.5 can be rewritten as following (Kay *et al.*, 1989b):

$$1/T_1 = S^2 \ (1/T_1)_{isot}\{[1 + (10 + \delta) / (3 + \delta)[(1 - S^2) / S^2](\tau_e/\tau_m)(\omega_X\tau_m)^2\} \qquad (2.7)$$

$$1/T_2 = S^2 \ (1/T_2)_{isot}\{1 + [10 + (7/6)\delta)] / (2 + (2/3)\delta)][(1 - S^2) / S^2](\tau_e/\tau_m)\} \qquad (2.8)$$

$$NOE = NOE_{isot} - 50/(3+\delta) \{[(1 - S^2) / S^2](\tau_e/\tau_m)(\omega_X\tau_m)^2\} \qquad (2.9)$$

where the subscript **isot** refers to the value in the absence of internal motion, and the value of δ = $(c/d)^2$. Therefore the order parameter S^2, effective correlation time τ_e and overall correlation time τ_m can be derived from fitting the above equations upon knowledge of the T_1, T_2 and NOE relaxation rate.

Two-dimensional ^{15}N relaxation (T_1, T_2, NOE) measurements of the L30 protein were recorded on Varian Inova 600 instrument. Series of spectra were acquired on a 0.7 mM protein sample at 10°C using published pulse sequences (Dayie & Wagner, 1994). Water flip-back was used for water suppression. The spectral widths used were 7000 Hz in F_2 (^1H) and 2000 Hz in F_1 (^{15}N). Sixteen scans were acquired for each t_1 increment; quadrature detection in the F_1 dimension was obtained using time-proportional phase incrementation (TPPI). A total of 1024 × 256 real data points were acquired for 6 different T_1 delays, corresponding to 10.02, 20.04, 40.08, 120.24, 300.6 and 601.2 ms for the T_1 measurements, and with repetition points of 10.02 ms and 601.2 ms. A 1.9 s relaxation delay was used between scans. T_2 measurements were recorded with the same spectral widths, number of scans, and data sizes as T_1 data, with 6 different T_2 relaxation delays, corresponding to 2.74, 5.48, 21.93, 41.11, 65.78, 104.15 ms, and repetition points of 2.74 and 5.48 ms. To eliminate the influence of chemical exchange on T_2 measurements, a Carr-Purell-Meiboom-Gill (CPMG) pulse train (Carr & Purcell, 1954; Meiboom & Gill, 1958) was used during the transverse relaxation time. Heteronuclear NOE (^1H-^{15}N) relaxation were measured using two spectra, one with the NOE effect, and one without. The duration of the recycling delay was 3 s per scan so as to ensure that the ^{15}N magnetization reaches its equilibrium value prior to the first ^{15}N pulse. The spectrum with NOE was obtained by proton saturation, which was achieved with a train of 120° hard pulses separated by a 5 ms delay for 3 s duration. The spectrum without NOE was recorded with identical parameters, except for the replacement of the 120° proton saturation pulses with a 3 s delay. The T_1, T_2, and NOE data sets were processed using Lorentzian-to-Gaussian apodization in both dimensions, and zero filling to 2048 (F_2) and 1024 (F_1) data points. Cross peak intensities (Barbato *et al.*, 1992) rather than

integrated peak volumes were measured in NMRPipe program (Delaglio *et al.*, 1995) and were used to determine the relaxation rates.

The T_1, T_2 data values were extracted by fitting cross peak intensities to a single exponential function, as follows:

$$I(t) = I(0) \exp(-t/T_{1,2})$$

where $1/T_{1,2}$ is the relevant relaxation rate and $I(t)$ is the intensity of a given cross peak at time t. More specifically, a conjugate gradient minimization was performed to determine the optimum value of the $I(0)$ and $T_{1,2}$ parameters by minimizing the χ^2 goodness of fit parameter:

$$\chi^2 = \sum [(I_c(t) - I_e(t)]^2/\sigma_I^2$$

where $I_c(t)$ are the intensities calculated from the fitting parameters, $I_e(t)$ are the experimental intensities, σ_I is the standard deviation of the experimental intensity measurements. The summation is performed over the number of time points recorded in each experiment. Errors in measured relaxation rates were estimated using Monte Carlo procedures (Farrow *et al.*, 1994).

The steady-state NOE values are determined from the ratios of the average intensities of the peaks with and without proton saturation. The standard deviation of the NOE values, σ_{NOE}, was determined on the basis of measured background noise levels using the following relationship:

$$\sigma_{NOE}/NOE = ((\sigma_{Isat}/I_{sat})^2 + (\sigma_{Iunsat}/I_{unsat})^2)^{1/2}$$

where I_{sat} and I_{unsat} represent the measured intensities of a resonance in the presence and absence of proton saturation, respectively, and σ_{Isat} and σ_{Iunsat} represent the standard deviations of the background noise. The root-mean-square value of the background noise regions was used to

estimate the standard deviation of the measured intensities. Spectra were recorded in duplicate to assess the validity of this estimate.

Figure 2.2.6 shows the relaxation parameters T_1, T_2 and NOE measured on the L30 protein. As can be seen, nine residues at the N-terminus and four residues at the C-terminus show significantly large T_2 rates and negative heteronuclear NOEs. This is an indication of a rapid motion (picosecond time scale). The observation of disordered terminal regions of protein is consistent with many results from similar studies on different other proteins (Farrow *et al.*, 1994; Kay *et al.*, 1989b). For the rest of the residues, the flexibility of the backbone residues are similar, except for G73. The large T_2 and negative heteronuclear NOEs of G73 could be a result of the flexible linkers (G72, G73, N74 and N75) connecting a β-strand and another secondary structure (not clear at this stage). While most of the measurements are reliable, residues between 74-78, and 81-88 have either no or less accurate relaxation rates that are either missing assignments or broad proton linewidths. The weak or missing correlation peaks are likely to be due to either conformational exchange or rapid amide exchange with solvent. The weak correlation peaks for these residues suggest these regions are relatively more flexible (millisecond time scale).

Furthermore, the correlation time τ_m was determined by the ratio of T_1/T_2 for each residue, and the average value and standard deviation of the ratio were also determined. The relaxation parameters of the residues having T_1/T_2 values within one standard deviation of the mean value and NOE values greater than 0.65 were fit to obtain τ_m for each residue. A very rough estimation of the global τ_m was made from the average of the individual values. The average overall correlation time τ_m was calculated to be 8.2 ± 0.4 ns for the L30 protein at 10°C. Since the overall correlation time of a molecule should be approximately proportional to its molecular weight, it was compared to calculated values for other proteins of similar size. In comparison with the correlation time of an SH2 domain complex (~ 6.5 ns for 13 kDa protein at 30°C) (Farrow *et al.*, 1994), the L30 protein shows a slightly higher value of 8.2 ns at 10°C. The discrepancy could be a result of different temperatures at which the experiments were recorded. Since the viscosity of solvent also increase with decreased temperature, therefore the overall
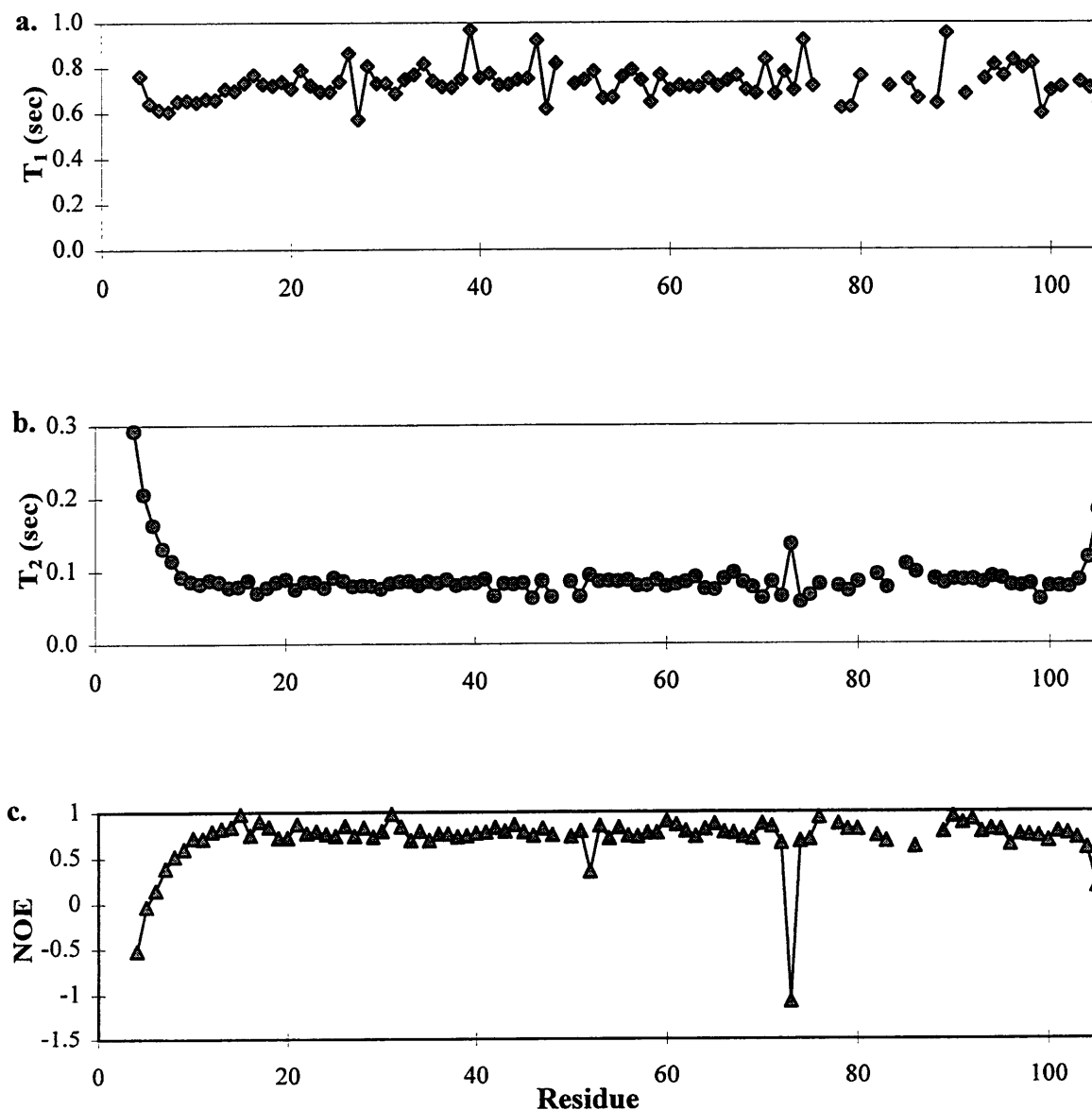
Figure 2.2.6: Plots of backbone relaxation parameters T_1, T_2, and NOE of the unbound L30 protein as a function of residue number. The data were recorded on a 600 MHz spectrometer at 10°C. Measured T_1 values are shown in panel **a**, T_2 values in panel **b**, and ^{15}N-^{1}H NOE data in panel **c**. The missing data points result from either broad correlation peaks or overlapping peaks in the ^{15}N-edited HSQC spectrum.

tumbling rate of L30 protein is slower at 10°C. A possibility could be the potential existence of a monomer-dimer equilibrium of L30 protein. Further measurements (*i.e.*, sedimentation equilibrium, light scattering) are necessary to investigate such speculation.

In conclusion, the biochemistry and NMR analyses of the L30 protein in the absence of RNA suggest that the protein adopts a structure with a mixture of both α-helices and β-sheets. The assignment of about 86% of the total observable proton resonances were achieved using the combination of both homonuclear and heteronuclear approaches. The analysis of sequential NOEs and chemical shift alignments of various backbone resonances facilitated mapping of the locations of three distinct α-helices and four β-strands within the L30 protein, with the exception of one flexible region (residues 74-88) of which the secondary structure is unclear at present. In addition, the initial studies of the dynamic properties of the protein reveal a possible flexible region (residues 74-88) that undergoes conformational exchange in the absence of its binding RNA. These studies of the L30 protein in the free form provide useful information, namely, resonance assignments, secondary structure maps and local conformational flexibility, which can assist in the later structural studies of the complex using NMR spectroscopy.

CHAPTER 3. The L30 RNA in the Free Form

Similar to the L30 protein in the free form, the studies of the L30 RNA in the absence of the binding counterpart are also essential for structural studies of the complex. In particular, the RNA under investigation is a large piece of premessenger RNA consisting of more than 1,000 nucleotides, but the functional regulatory regions reside at the 5' end of the exon and few residues within the intron. In order to make the target RNA amenable for structural studies using NMR spectroscopy, it is necessary to design a model system to reduce the size of the molecule based on the biochemical analyses.

3.1. Design of L30 RNA for NMR

The NMR spectrum of an RNA is much more crowded than that for a protein. Thus, for a feasible analysis it is necessary to have as small RNA as possible. When combined with the desire to determine the structure of the RNA bound to a protein, as is the case presented here, the design is mainly based on minimizing the size without losing binding affinity.

The L30 RNA design was based on MiniL32 (Li *et al.*, 1995), a small stem-loop-stem RNA that has been used as a model system for biochemical analyses of the L30 protein pre-mRNA binding site for the auto-regulation of splicing. The MiniL32 RNA that was designed to mimic the phylogenetically derived stem-"purine rich loop"-stem structure has been subjected to extensive chemical and enzymatic probing studies. Results from RNase footprinting, chemical modification interference, and studies of protection on protein binding suggested that several purine bases in the internal loops are critical for protein binding (Li *et al.*, 1995) (Figure 3.1.1, panel **a**). More recently, *in vitro* selection experiments on RNA aptamers were carried out, and they also show the preservation of the wild type stem-internal loop-stem secondary structure (Li & White, 1997) (Figure 3.1.1, panel **b**). In particular, a two-plus-five internal loop that is rich in purines appears to be conserved and may be important for protein binding. These biochemical characterizations of the pre-mRNA autoregulatory binding site have helped in improving upon

(a)

```
            U
      U          A
    U          U   50
        U  –  A
  15  G  –  C
       U  –  G
      (G) – C
     (A)       (A) (G)  57
    (G)             A
  10  G           (G)
                  (A)
       C  –  G   U
       C  –  G
       A  –  U
       G  –  C
            A  65
      g     G
      g         3'
     g
  5'
```

MiniL32 RNA

(b)

```
       c    a
    g          a  21    Tetraloop
       c  –  g  50
       U  –  A
  15   G  –  C
       U  –  G          Upper stem
       G  –  C
      A        A   G
    G             A  57
                  G    Internal loop
  10  G           A
       C  –  G   U
       C  –  G
       A  –  U          Lower stem
       G  –  C
       g  –  c  65
    5'          3'
```
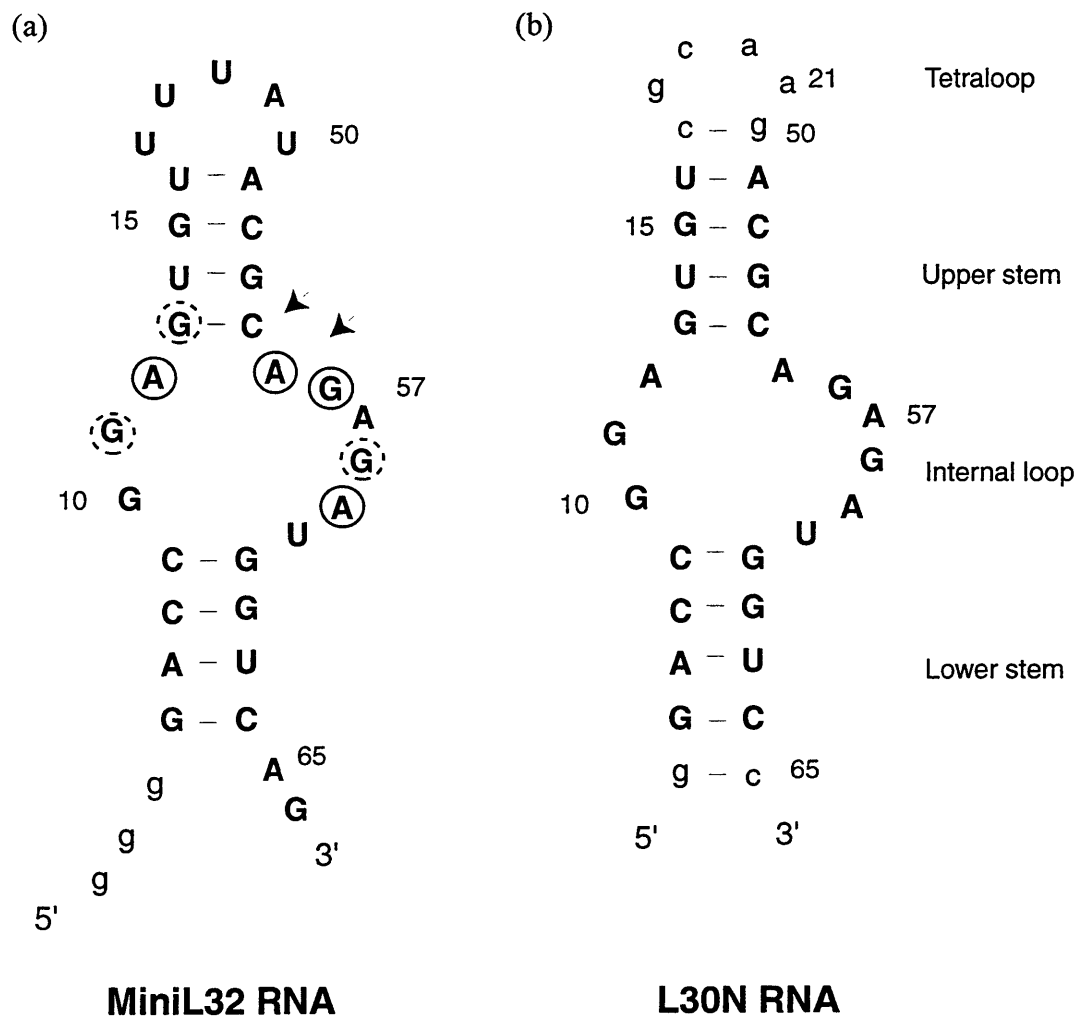
L30N RNA

Figure 3.1.1: Secondary structures of **a**, MiniL32 RNA used in biochemical analyses and **b**, L30N RNA used in NMR structural studies. In the MiniL32 RNA, circles (open or dashed) and arrows represent chemical modifications of bases and phosphates, respectively, that are important for L32 protein binding. Open circles represent nucleotides protected from cleavage upon protein binding. Lower case letters represent non-native nucleotides. The sequence of the L30N RNA used for NMR analysis is modified from the MiniL32 RNA: a G-C pair was added to stabilize the lower stem and replace the unpaired ends, and a GNRA tetraloop was used to replace the pentaloop at the upper stem and prevent dimerization of L30N RNA in NMR studies.

MiniL32, though more biochemical experiments had to be carried out for designing a suitable RNA model system for studying the structure and function of the L30 protein-RNA complex using NMR spectroscopy. A complete description of how the RNA was designed is given in the following subsections.

3.1.1. Structure mapping and design of RNA for NMR

3.1.1.1. Problems with MiniL32 RNA

As described above, the pre-mRNA autoregulatory binding site of the L30 protein can be best represented by a 36 nucleotide MiniL32 RNA used in biochemical characterizations (Li *et al.*, 1995) (Figure 3.1.1, panel **a**). Despite having a reasonable size, MiniL32 RNA was not an ideal model system for NMR analyses mainly of problems arising from the sequences selected for the lower and upper stems. More specifically, three Gs that were added for facilitating transcription create single stranded regions at both the 5' and the 3', while the chosen non-wild type loop rich in Us at the upper stem leads to thermal instability. Initial NMR studies on MiniL32 showed that the conformational flexibility at these ends lead to unfavorable exchange properties making it not suitable for NMR analyses (data not shown).

The conformational heterogeneity of MiniL32 made it necessary to modify the sequence. Initially the *in vitro* selection results were not available, therefore slight modifications were carried out to tailor the MiniL32 RNA for NMR studies. Later these regions were also found to be unimportant for L30 protein binding (Li & White, 1997). First, as alluded to above, the single stranded regions at the 5' and 3' ends were eliminated, and a G-C pair was added to stabilize the lower stem and facilitate T7 polymerase transcription. Second, the upper closing loop was replaced with GAGA (a GNRA-type tetraloop) loop. The preliminary analysis from a 2D NOESY spectrum suggested that there was a substantial amount of conformational and/or structural heterogeneity in this RNA sample. These was reflected by several sets of exchange peaks (with about 15% of a minor conformer) present in the imino resonance region. This

heterogeneity was also seen in the gel shift analysis and size exclusion chromatography, which showed a substantial amount (> 10%) of dimerization.

3.1.1.2. Preliminary mapping

The failure to design a conformationally stable RNA from the above results led not only to various other modifications and biochemical characterizations of MiniL32, but also to reinterpretations of the above studies. The first set of experiments was carried out to understand the exact base pairing in the asymmetric, purine rich loop. *In vitro* SELEX experiments (personal communication with Dr. Susan White) showed that the phylogenetically conserved A57 was always replaced by a cytosine. Such a replacement could in principle lead to an internal loop conformation resembling the Loop-E motif (Dallas *et al.*, 1995). When the sequence of the L30 pre-mRNA was compared to the Loop-E motif, it suggested the formation of a long continuous helix extended by wobble base pairs, rather than the stem-loop-stem conformation predicted from the phylogenetically related species (Eng & Warner, 1991).

To further check this prediction of a Loop-E motif conformation and to design a more stable RNA, a series of modified L30 RNA were made as summarized in Figure 3.1.2. Panel **a** of the figure is divided into two sections. The left side refers to modifications on the L30R RNA in which the loop in the MiniL32 is replaced by stable tetraloops, and a G-C pair was added at the 5'- and 3'-ends. The RNAs on the right side of the panel are different types of modifications, in which a tetraloop is used to stabilize the left stem and a G-C pair is added to the right stem. The general rationale for the modifications was to maximize the stability of the RNA monomers, which could be tested later by size exclusion chromatography at the millimolar NMR concentration. Among the RNAs listed in Figure 3.1.2, some were drawn as almost straight helices, resembling the Loop-E motif. These were used to test the hypothesis that the L30 RNA might adopt a Loop-E motif conformation rather than the predicted two-plus-five internal bulge loops.

a.

MiniL32

```
            G G A
5'  gGACC G     GUGU U U U
3'  cCUGG       CGUA   A
         U    A     U
          A  G
           G A
```

L30/33

```
              G G A
u g GGACC G      GUGU c c  3'
u u CCUGG        CGUA g g  5'
           U    A
            A  G
             G A
```

L30N

```
            G G A
5'  gGACC G     GUGU c g c
3'  cCUGG       CGUA g
         U    A      a
          A  G
           G A
```

L30/37

```
c g GGACCGGAGUGUUUAc c  3'
u u CCUGGUAGAACGCAUg g  5'
                  G
```

L30R

```
            G G A
5'  gGACC G     GUGU g
3'  cCUGG       CGUA   a
         U    A     a
          A  G       g
           G A
```

L30/31C

```
c g GGACCGGAGUGUc c  3'
u u CCUGGUAGcGCGg g  5'
                A
```

L30/29

```
5'  gGGACCGGAGUGc u u
3'  cCCUGGUAGAACG   c
                 G  g
```

L30/33C

```
              G G A
c g GACC G       GUGU c c  3'
u u CUGG         CGUA g g  5'
          U     A
           A  G
            G c
```
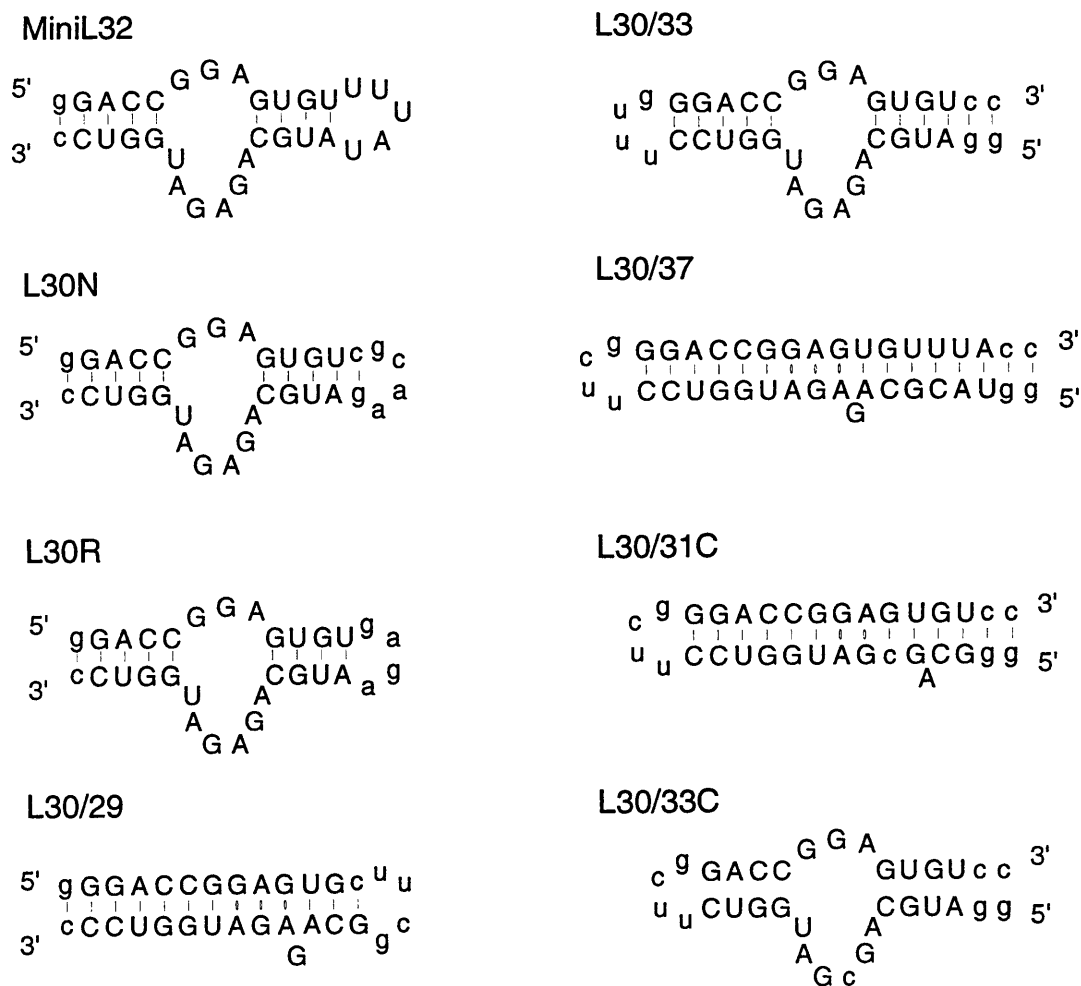
b.

RNA	MiniL32	L30N	L30R	L30/29	L30/33	L30/37	L30/31C	L30/33C
Binding to MBP-L30 K_d (nM)	14	12	23	1025	15	14	2139	18

Figure 3.1.2: The sequences of the RNAs and their binding affinities for MBP-L30 fusion protein. Panel a shows the secondary structures of the RNAs used for mapping the internal loop structure for L30 protein binding. Lower case letters indicate the modified base sequences. The secondary structures are drawn based on the putative base pairing for the sequences. Panel b shows the RNA binding affinities for the MBP-L30 fusion protein determined from gel shift binding assays.

All these RNAs were tested for the binding activities to the MBP-L30 fusion protein. Their dissociation constants (K_ds) are listed in Figure 3.1.2, panel **b**. It is clear from the protein binding data that RNAs that adapt the two-plus-five internal bulge loop conformation have close to wild type binding activity. On the contrary, the alternative hypothesis that the RNA might adopted Loop E conformation was not consistent with the binding data. Both L30/29 and L30/33C show almost a 500- to 1000-fold reduction of binding affinity to the MBP-L30 fusion protein. This suggests that the Loop-E conformation, stabilized by the continuous helix formation, is unfavorable for binding. Even though L30/37C is expected to exist in a Loop-E conformation, the loose base pairing at the right side of the RNA allows base pairs to rearrange upon protein binding, ultimately resulting in the near wild type binding activity. Among the RNAs tested, L30N representing the best model system for studying the structure of the L30 protein pre-mRNA binding site and has the optimum activity.

3.1.1.3. Useful RNA mutants

In addition to designing a good model system for RNA structural analyses, mutants were also synthesized at different stages to facilitate NMR experiments. These mutants were made mainly because of the difficulties encountered in the resonance assignments in the internal loop region of L30N RNA. As an example, there are two putative wobble U·G and G·U pairs (G10-U60 and U14-G53) in the RNA. Ambiguities were raised when one base pair was constantly observed in the NMR spectroscopy at all temperature but the other one was seen only at low temperatures. It was known from the SELEX results (Li & White, 1997) and from phylogenetic comparisons (Eng & Warner, 1991) that the importance of U14·G53 was less significant, and thus it was possible to use a Watson-Crick base pair substitution without affecting the overall binding affinity to the protein. Therefore, a U14C mutant leading to a Watson-Crick pair at positions 14 and 53 was prepared to differentiate between the two wobble pairs. Other point mutants (A57C, A55C and A55U) were also designed according to SELEX results in order to facilitate the NMR assignments. This will be discussed in detail in Chapter 5. To ensure that the modifications in the RNA did not affect the protein binding, gel shift protein binding assays were carried out to determine the RNA binding affinities and stoichiometries to the L30 protein as

described previously in Chapter 2. All the RNAs had a protein binding affinity that was comparable to MiniL32 RNA. The near wild type MBP-L30 protein binding affinity (K_d ~ 14 ± 2 nM) suggested that these RNA mutants would be valuable assistants in the structural analyses.

3.1.2. Size-exclusion chromatography assay

To monitor potential dimer formation at NMR concentrations, RNAs were analyzed on a Vydac size-exclusion chromatography column equilibrated with the standard NMR buffer (10 mM sodium phosphate, pH 6.5, 50 mM NaCl, and 0.1 mM EDTA). Briefly, RNAs ranging from 10 to 1000 μM were heat denatured at 65°C for one minute, and then flash cooled on ice for 5 min. RNA aliquots of 2 μl were injected into a Vydac column at a flow rate of 0.5 ml/min and the peaks were monitored by UV absorption at 260 nm. The retention time of the monomer was estimated by comparison to that of a previously measured monomeric RRE RNA (34 nucleotides), and it was further standardized by the retention times of protein markers with known molecular weights. The retention time for a dimer is expected to be less than that of a monomer, therefore the population of monomer versus dimer can be determined by the ratio of the areas under the two peaks.

Most RNA constructs used for the biochemical studies were subjected to the size-exclusion chromatography assay. Among these RNAs, L30R RNA displayed about 15-20% dimerization at a relatively lower RNA concentration (0.5 mM), consistent with the earlier NMR observation on the free RNA. A majority of the RNAs with an extra-stable tetraloop at one end and two G-C closing pairs at the other end exhibit over 90% monomer conformation at millimolar concentrations.

3.2. RNA Synthesis

3.2.1. Preparation of $^{13}C/^{15}N$ nucleoside triphosphates (NTPs)

To facilitate the NMR assignments of the RNA, it was necessary to prepare doubly labeled RNA to help in resolving many ambiguities, similar to that in the case of the protein as described in Chapter 2. With the recent developments in preparing isotopically labeled nucleotides (Batey *et al.*, 1992), it is now possible to prepare $^{13}C/^{15}N$-labeled samples. The basic protocol can be divided into two steps: preparation of NTPs and transcription into RNA. The NTPs were prepared and purified as described by Batey *et al.* (1992). A brief description of the various steps in the protocol is given below.

Cell growth: Ten liters of *Methylophilus methylotrophus* cells were grown in phosphate media (1.9 g/L K_2HPO_4, 1.79 g/L $NaH_2PO_4 \cdot H_2O$, 5.1 mg/ml $FeCl_3$, 0.81 mM $MgSO_4$ and 1× trace metals, pH 7.0) containing 1.75 g/L $(^{15}NH_4)_2SO_4$ and 10 ml of ^{13}C methanol as the sole nitrogen and carbon source, respectively. Cells were supplemented with four additional aliquots of 10 ml methanol when the cell density measured by OD_{660} reached 0.4, 0.8, 1.2 and 1.6, and were harvested when the OD_{660} reached 2.6. This procedure yielded about 61 grams of $^{13}C/^{15}N$ *Methylophilus methylotrophus* cells.

Preparation of ribose nucleoside monophosphates: About 16 grams of cells were lysed in STE buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 0.5% SDS and were extracted with phenol/chloroform to separate nucleic acids from protein. The isopropanol-precipitated nucleic acids were then subjected to nuclease P1 digestion in a buffer containing 15 mM sodium acetate, pH 5.3, and 0.1 mM $ZnSO_4$ at 65°C for 12 hrs. Then the ribonucleoside monophosphates (NMPs) were separated from deoxyribonucleoside monophosphates (dNMPs) using boronate affinity chromatography. This column selectively binds the cis-diols of the ribose ring at pH 9.5, maintained by using 1 M triethylammonium bicarbonate (TEABC) buffer, and

can be eluted by acidified solution (carbonate water, pH 4.6). An RNA/DNA ratio of 3 was observed.

Preparation of ribose nucleoside triphosphates: Previously, instead of having buffer for stabilizing the pH of the solution, diluted HCl (0.1 N) was added occasionally through a peristaltic pump during the course of the charging reaction to neutralize the rising pH. As a result, the final volume of the reaction increased by about 30-40% compared to the original volume. In the present case, potassium phosphate buffer was chosen for maintaining the pH of the charging reaction. In buffer containing 50 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, 15 mM dithiothreitol (DTT), 10 mM sodium 3'-phosphoglycerate, NMPs were enzymatically converted into NTPs. The following enzymes were used: phosphoglycerate mutase, nucleoside monophosphate kinase, enolase, myokinase, pyruvate kinase and guanylate kinase. Complete phosphorylation of all nucleotides took about 36 hours, and the reaction was monitored by Vydac nucleoside HPLC chromatography (Figure 3.2.1.). The NTPs were further purified for transcription with another round of boronate affinity column chromatography.

3.2.2. Preparation of the RNAs for NMR characterization

The following RNAs were prepared at various stages in the NMR studies (the nucleotides in bold are wild type sequence, and mutations are underlined):

L30N (wild type): 5'-**GGACCGGAGUGUCGCAAGACGCAGAGAUGGUCC**-3'

L30R (wild type): 5'-**GGACCGGAGUGUGAGAACGCAGAGAUGGUCC**-3'

L30U$_{14}$C (mutant): 5'-**GACCGGAGC̲GUCGCAAGACGCAGAGAUGGUC**-3'

L30A$_{55}$C (mutant): 5'-**GGACCGGAGUGUCGCAAGACGCC̲GAGAUGGUCC**-3'

L30A$_{57}$C (mutant): 5'-**GGACCGGAGUGUCGCAAGACGCAGC̲GAUGGUCC**-3'

L30A$_{57}$U (mutant): 5'-**GGACCGGAGUGUCGCAAGACGCAGU̲GAUGGUCC**-3'

GMP
AMP
UMP

CMP

GMP
UMP
CMP
ATP

ATP
GTP
UTP

ATP
GMP GDP UTP GTP
ADP
CTP

CTP

0 hr

3 hrs

21 hrs
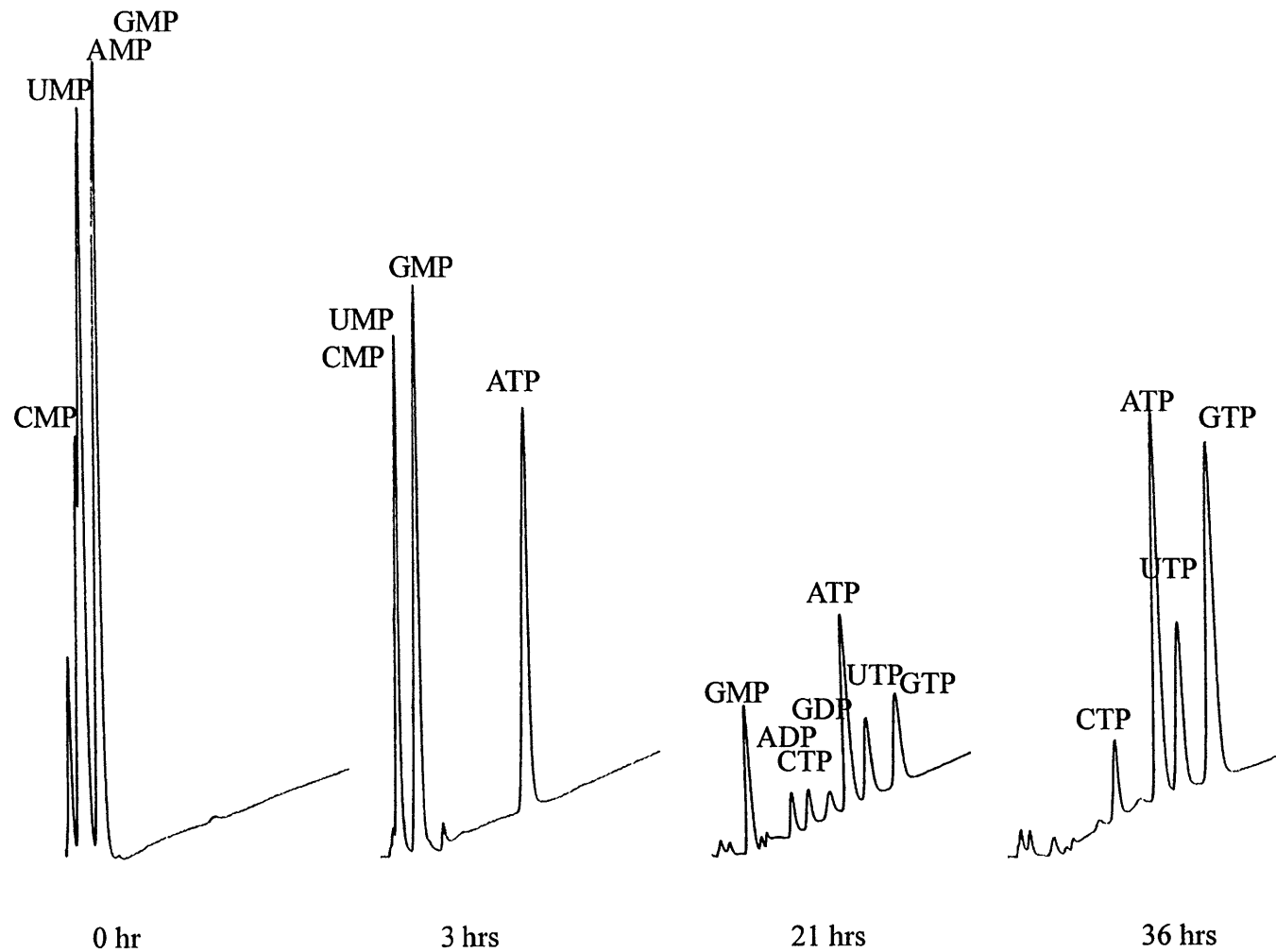
36 hrs

Figure 3.2.1: HPLC traces of the time course during enzymatic conversion of nucleotide monophosphates (NMP) into nucleotide triphosphates (NTP). An almost complete conversion of NMP into NTP occurred within 36 hrs.

The above RNAs were synthesized by phage T7 RNA polymerase transcription using different DNA templates containing identical double-stranded promoter regions and different single-stranded anti-sense coding regions (Wyatt *et al.*, 1991). The 18 nucleotide DNA promoter sequence ("top strand") used is 5'-CTAATACGACTCACTATA-3'. The individual DNA templates contains 5'-anti-sense coding sequences plus 5'-TATAGTGAGTCGTATTAG-3'. Transcription were performed in 80 mg/ml polyethylene glycol, 1× transcription buffer (80 mM HEPES, pH 8.1, 10 mM DTT, 1 mM spermidine, 0.01% triton X-100), with 300 nM DNA template, 300 nM T7 DNA top strand and 0.5 unit/ml inorganic pyrophosphatase. NTPs, $MgCl_2$ and T7 RNA polymerase concentrations were optimized for highest yields on the different DNA template (Table 3.1). Optimizations were carried out in 20 µl reactions containing trace amounts of [α-^{32}P] labeled GTP, and the full-length RNA products were quantified by phosphorimaging. Conditions that gave the maximum amount of full-length RNA per NTP input were chosen for large-scale RNA sample preparations.

A 50 ml transcription using the optimized condition generally yielded five to ten milligrams of RNA. More specifically, after the enzymatic synthesis at 37°C for 3.5 hrs, the crude reaction mixture was extracted with an equal volume of phenol, followed by extraction using a chloroform and isoamyl alcohol mixture (24:1 ratio). It was then allowed to precipitate overnight precipitation using three volumes of ethanol and 0.1 mM sodium acetate (pH 5.5) at -20°C. The crude RNA mixture was then purified using preparative gel (45 × 35 × 0.3 cm^3) electrophoresis on a 20% (w/v) polyacrylamide (19:1) denaturing gel containing 8M urea. The product band was excised from the gel under UV shadowing. The RNA from the gel was recovered by electro-elution in an Elutrap apparatus (S&S), followed by ethanol precipitation of the electroeluant. The RNA pellet was completely suspended in 400 µl ddH$_2$O and dialyzed extensively against the NMR buffer (10 mM potassium phosphate, pH 6.0/6.5, 0.02% NaN$_3$, and 0.1 mM EDTA). The concentration of RNA was measured by UV$_{260}$ absorbance and calculated using the conversion factor 1 OD$_{260}$ = 35 ng of RNA. A 50ml transcription yielded a 1.5 mM to 2.0 mM NMR sample. Variations depended on the RNA sequence and the T7 enzyme activities. Unlabeled RNAs were prepared using the above protocol with NTPs purchased from Sigma;

Table 3.1: Optimized reagent conditions of T7 RNA polymerase transcription for all RNAs used in NMR experiments. All reactions were carried out in buffer containing 80.0 mM HEPES, pH 8.1, 10 mM DTT, 1.0 mM Spermidine, 0.01% (v/v) Triton X-100, 80 mg/ml PEG-8000, 300 nM DNA template, 300 nM T7 DNA promoter and 1 unit/ml inorganic pyrophosphatase. Characters in bold indicate $^{13}C/^{15}N$-labeled nucleotide triphosphates.

Reagent	L32N	L32R	U14C mutant	A55C mutant	A57C mutant	A57U mutant	Uniformly $^{13}C/^{15}N$ labeled L32N	Selectively $^{13}C/^{15}N$ labeled L32N (guanosine)
ATP (mM)	4.0	4.0	4.0	3.5	3.5	3.5	**2.0**	2.0
GTP (mM)	6.0	4.0	6.0	6.0	6.0	6.0	**2.0**	**2.0**
CTP (mM)	4.0	4.0	4.5	4.5	4.5	4.0	**2.0**	2.0
UTP (mM)	2.0	4.0	1.5	2.0	2.0	2.5	**2.0**	2.0
MgCl$_2$ (mM)	25.6	25.6	25.6	25.6	25.6	25.6	8.0	9.6
T7 RNA polymerase (mg/ml)	0.035	0.035	0.035	0.035	0.035	0.035	0.025	0.025

^{13}C/^{15}N-labeled RNAs were prepared using the NTPs purified from the *Methylophilus methylotrophus* as described above.

3.3. NMR Characterization of the L30N RNA in the Free Form

3.3.1. Optimization of the experimental conditions

Initially the NMR conditions were optimized solely based on the stability of the RNA in the absence of its cognate protein, and all NMR spectra were recorded at 15°C in a standard NMR buffer (50 mM NaCl, 10 mM sodium phosphate, pH 6.5, and 0.1 mM EDTA). Later it was determined that this buffer condition was not optimal for studying the L30 RNA-protein complex structure. The complex proved to give sharper signals at a lower salt concentration (10 mM monovalent ions) and higher temperature (30°C). Moreover, for the L30 protein in the complex, amide protons show much slower exchange rate with solvent around pH 5.5, which has greatly facilitated the triple resonance experiments carried out for backbone sequential correlations. In contrast to this, the RNA imino proton resonances broaden when the pH drops below 6.0 (both in the free and the complex form). Thus, the RNA data in the complex was collected in NMR buffer containing 10 mM potassium phosphate, pH 6.0, 0.02% NaN$_3$ and 0.1 mM EDTA. Temperature dependence (from 5°C to 25°C for free RNA) was used to resolve ambiguities associated with resonance overlaps and exchange broadening. All RNA complexes were dialyzed into pH 6.5 buffer for comparison with the free RNA.

3.3.2. Assignment of exchangeable proton resonances

Among the NMR experiments recorded for RNA structure analysis, homonuclear NMR experiments played a major role in these structural studies. Besides, isotopic labeling of RNA is much more expensive and a labor-intensive procedure (see section 3.2.1) compared to protein. Moreover, many heteronuclear experiments did not provide much useful information, especially those associated with aromatic rings, because of relaxation properties. For the mutants,

homonuclear experiments were solely sufficient to provide useful information by following the changes in the imino resonance spectra, or the appearance of H5-H6 cross peak upon mutation of purine to pyrimidine in D_2O NOESY and COSY spectra. Nevertheless, the assignment procedure did required a combination of both homonuclear and heteronuclear experiments.

3.3.2.1. Imino resonance assignment

For observing exchangeable proton resonances, 1D spectra of L30N RNA dissolved in 90/10% H_2O/D_2O were recorded on a Varian VXR-500 MHz instrument using 1331 binomial pulse sequence (Hore, 1983), or on a Varian Inova-600 MHz spectrometer using WATERGATE sequence (Mori *et al.*, 1995) for water suppression. The imino protons of G's and U's in base pair conformations resonate quite downfield (between 12 to 15 ppm) and usually provide an estimate regarding the presence of base pairing. Thus, the imino resonances are a direct indication of structure formation in the RNA. The imino-proton regions of L30N RNA recorded at different temperatures were compared and used to optimize the resonance resolution and signal intensity. When the temperature was increased from 5 to 15°C, no significant changes in the imino resonances were observed. On the contrary, when the temperature was raised from 15°C to 30°C, some resonances sharpened as expected, but some broadened. The broadening occurs mainly because of the melting of the RNA. Resonances between 10 to 12 ppm broaden first, since they belong to the unpaired G imino protons, which experience exchange as the temperature is increased. Temperatures of 10, 15 and 25°C gave better resolution and signals in 1D imino proton resonance region were chosen to record 2D spectra for assignment of the imino resonances.

Two-dimensional NOESY-WATERGATE experiments with 100 ms and 150 ms mixing times were acquired on the RNAs in water on either the Bitter 500 MHz or Varian 600 MHz spectrometer. The WATERGATE delay was set to 110 μs to maximize the signals in the imino resonance region. Data sets with 4096 complex points in t_2 and 512 complex points in t_1 were acquired with 12000 Hz (on the 500 MHz machine) or 14000 Hz (on the 600 MHz machine) spectral widths in both dimensions. A ROESY-WATERGATE experiment with a 50 ms mixing

time was acquired using similar acquisition parameters on the Varian 600 MHz spectrometer, except that the additional spin-lock period was achieved with a 7 kHz spin-lock pulse. The 2D NOESY spectra provide direct information on the base pairing and the sequential base pair connectivities (Wüthrich, 1986). Typically a Watson-Crick G-C (or C-G) pair is identified by the strong NOE cross peaks from the two amino protons of cytosine to the guanine imino proton, whereas a U-A (or A-U) pair is identified through a sharp cross peak between the uracil imino proton and the adenosine H2 proton. For temperatures greater than 10°C, the amino protons of both adenine and guanine are not observable when they are in Watson-Crick base pairs as there exists a slow rotation about the N6-C6 bond upon base pairing. Besides the two types of Watson-Crick base pairs, a wobbled G·U (or U·G) pair can be identified by strong intra-base pair imino-imino NOEs, and imino-amino proton NOEs. Based upon these characteristic patterns, two A-U/U-A pairs, six G-C/C-G pairs, and one U·G pair were observed in L30N RNA in the free form at 15°C. An additional G·U pair was observed in spectra acquired at 10°C, which disappeared with rising temperature (Figure 3.3.1).

The initial homonuclear NOE-based categorization of G and U imino resonances was further confirmed by a ^{1}H-^{15}N HSQC spectrum acquired on a fully ^{13}C/^{15}N-labeled RNA. Standard ^{15}N-edited HSQC (Mori *et al.*, 1995) spectra at 11, 15 and 25°C were acquired by either centering the ^{15}N transmitter near 117 ppm to obtained ^{1}H/^{15}N correlation for the entire exchangeable resonances or around 150 ppm for observing only the imino ^{1}H/^{15}N correlation. The evolution period was optimized for a J_{HN} = 90 Hz. In a ^{15}N-edited HSQC spectrum, the G H1 can be easily distinguished from the U H3 by the more down-field chemical shift of its attached ^{15}N resonance (Figure 3.3.2). 12 G's and 4 U's imino resonances were observable in ^{15}N-HSQC spectra acquired at 10°C, although there are a total of 13 G's and 4 U's in the sequence. These imino resonances include 7 G's and 4 U's that form base pairs, one G involved in a tetraloop base pair, and 4 others within internal loop which was not yet assigned. The missing imino must belong to the G5 at the 5'-end, which is expected to broaden out.

By analyzing the sequential weak imino-imino NOEs between stacked base pairs, the base pairs could be grouped into two segments corresponding to the two predicted helical stems.
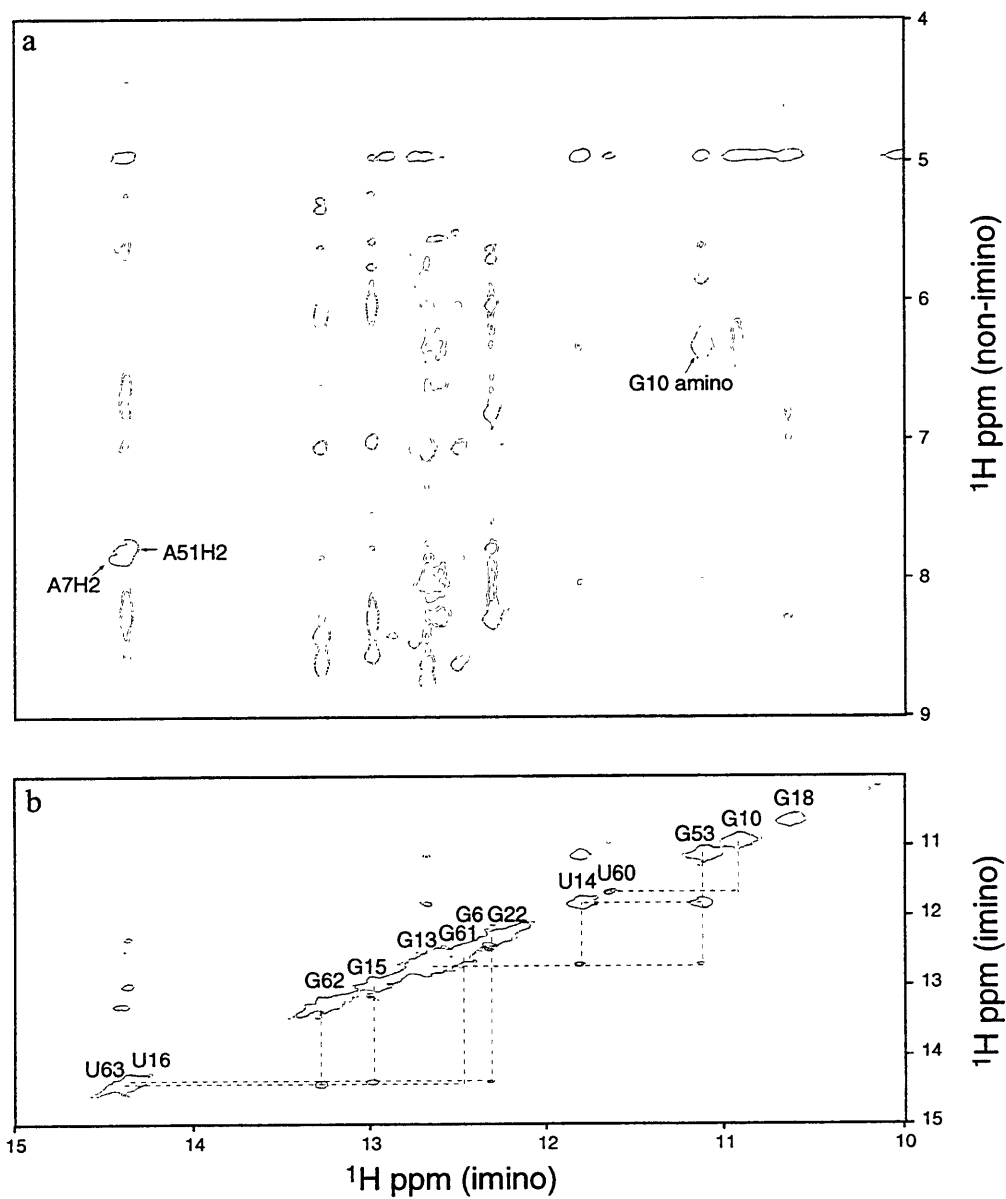
Figure 3.3.1: Assignment of imino proton resonances. Imino NOEs observed for regions of **a**, non-imino (*i.e.*, aromatic, amino and sugar proton) resonances, and **b**, imino resonances of L30N RNA in 90/10% H_2O/D_2O at 10°C. The dashed lines trace the sequential imino-imino NOEs between the stacked base pairs.
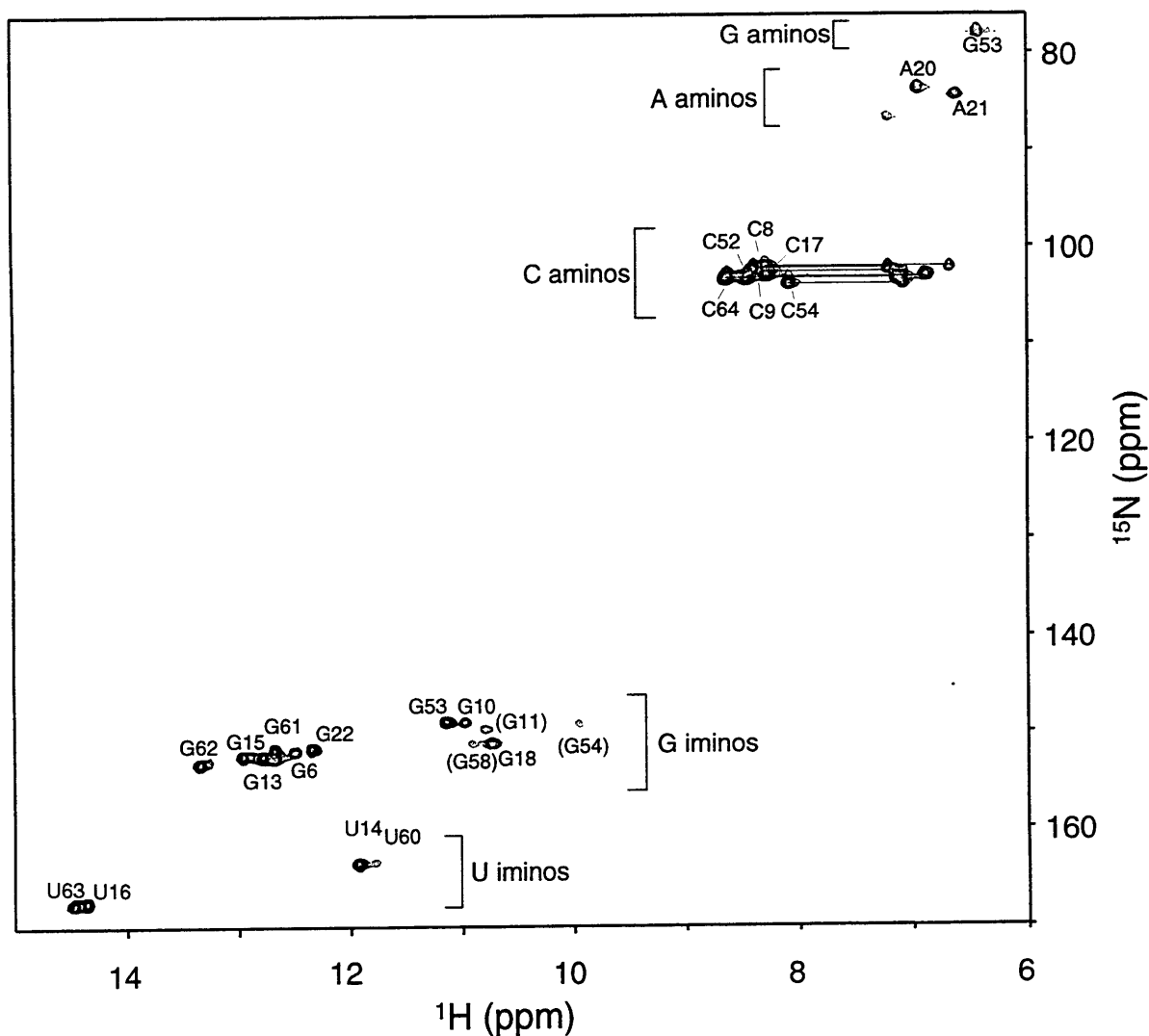
Figure 3.3.2: ^{1}H-^{15}N HSQC spectrum of L30N RNA. The spectrum was recorded in 10 mM potassium phosphate buffer, pH 6.0, containing 0.02% NaN$_3$ and 0.1 mM EDTA at 10°C. Peak assignments are given, and resonance types characterized by chemical shift ranges are denoted by brackets. The bases in parentheses are tentative assignments.

While most imino proton resonances of the G-C and A-U pairs within each helix were assigned on the basis of sequential NOE analyses, resonances associated with wobble G·U pairs could not unambiguously identified. There are two G·U pairs in the sequence: the G10·U60 pair at the end of internal loop and the U14·G53 pair in the helical stem. Only one wobble G·U pair showed a strong intra-base imino-imino NOEs at 15°C NOESY spectrum; the other one appeared only at lower temperature. The latter was assigned to the G10·U60 pair, because exchange broadening is anticipated for nucleotides at a helical end. But there is a slight ambiguity with this assignment. While the stronger pair (assigned to U14·G53) of imino resonances only gave sequential imino-imino NOEs to one G-C pair, two sequential NOEs are expected from the sequence prediction, since there the U14·G53 is sandwiched between two G-C pairs. According to phylogenetic comparisons and biochemical analyses (Eng & Warner, 1991; White & Li, 1995; Li & White, 1997), nucleotides G10 and U60 are expected to be an important base pair for protein recognition, whereas the U14·G53 pair should not be so important and can be replaced with a Watson-Crick base pair. To further support the resonance assignments, a mutant replacing the U14 with a C thereby creating a C-G pair was made. As expected, the strong resonance peaks corresponding to the U14·G53 pair disappeared, and peaks corresponding to a G-C pair appeared, which also showed similar NOE connection to the adjacent base pairs. This confirmed the assignments for the U14·G53 and the G10·U60 base pairs.

Weak sequential imino-imino NOEs connectivities were observed for all the stacked base pairs with one exception. No connection was seen between U14·G53 and G15C52, indicating a unique stacking arrangement adopted by the G·U pair. In an A-form helix, a G·U pair is nearly unstacked from the base pair 5' to the guanine, but it stacks well on the pair to the 3' side of the guanine. This stacking arrangement leads to the pattern of a highly twisted dinucleotide step followed by a step with low twist (Allain & Varani, 1995). Using the sequential NOE connectivities approach, imino resonances from an A-U and three G-C (or C-G) pairs were assigned in the lower stem; and an U-A, an U·G and three G-C (or C-G) pairs were assigned in the upper stem. Weak imino resonances of a sheared GA pair in the tetraloop and the G10·U60 wobble pair were also assigned using spectra recorded at lower temperature. Figure 3.3.1 shows the sequential NOE walk of the imino region in a NOESY spectrum acquired at 15°C.

The inter-base NOE connectivities were interrupted at the internal loop region. The imino protons in the internal loop (G11, G54, G56 and G58) could be observed but had broad linewidths and narrow resonance dispersion (between 10.0 and 11.0 ppm) in ^{15}N-edited HSQC spectra acquired at low temperature, indicative of conformational exchange and absence of hydrogen bonds in this region. As a result, these resonances did not give rise to observable NOEs in the NOESY experiment, and no specific assignments were made for these imino protons.

3.3.2.2. Amino resonance assignments

After completing the assignments of guanine imino resonances, identification of the cytosine amino protons was straightforward. There are 8 cytosine in the L30N RNA sequence, 7 of them are involved in Watson-Crick base pairing, and the other one is located within the tetraloop. Six pairs of amino protons were identified through the characteristic strong NOEs to directly paired G imino proton resonances. The C65 residue at the 3'-terminus and C19 in the tetraloop were not identified as they showed no cross peaks in either NOESY or ^{15}N-edited HSQC spectra. The NOE-based assignment of cytosine amino proton resonances was confirmed by the ^{15}N-edited HSQC spectrum, which shows pairs of amino protons at the attached ^{15}N frequency (around 100 ppm). Identification of cytosine amino proton resonances not only provided evidence for Watson-Crick base pair formation, it was also important for assisting in the assignment of nonexchangeable protons, as will be discussed later.

Purine amino proton resonances on the other hand, showed fewer observable cross peaks in NOESY and ^{15}N-edited HSQC spectra. Exocyclic amino protons of most of the purines involved in hydrogen bonding conformation were not observable under the current experimental condition. This was primarily due to intermediate exchange rotation along C-N bonds, but the cross peaks corresponding to the amino protons that were not involved in hydrogen bonds were observed. Within the two helical regions, a strong NOE from the exocyclic amino proton was also observed for the G53 in the G53-U14 wobble pair. This was also expected because of free

rotation about the C2-N2 bond in the absence of hydrogen bonding. Similar NOE was also seen for the G10 amino resonances in the G10-U60 pair, but with relatively weaker intensity. In addition to observable guanine amino protons, a couple of adenosine amino protons within the tetraloop were also observed, consistent with the established tetraloop assignments (Battiste, 1996). Exocyclic amino protons of guanines were distinguished from those of adenosines on the basis of different chemical shifts of the attached ^{15}N in an ^{15}N-HSQC spectrum. Although most Watson-Crick paired purines displayed no noticeable NOEs from the amino protons in a NOESY spectrum, the G9-C22 pair that flanks the upper internal loop showed NOEs not only from the amino protons of C22 but also from the two distinctive amino protons of G9. These amino protons showed strong NOEs to its own imino protons and the amino protons of C22. This type of NOE pattern has been previously observed in the AMP-aptamer (Jiang *et al.*, 1996), and can be explained by a more frequent "breathing" that occurs at the terminal base pair. None of the purines within the internal loop exhibited identifiable cross peaks. Conformational averaging within the internal loop rather than extensive hydrogen bonding between these purines accounts for the broad linewidth. Overall, only a few G's and A's amino protons were assigned in the L30N RNA sequence.

3.3.3. Assignment of the stem nonexchangeable resonances

The assignment of the nonexchangeable protons in the L30N RNA was made using a range of homonuclear and heteronuclear multidimensional NMR experiments. The strategy included initial analyses of two-dimensional homonuclear NOESY, COSY and TOCSY spectra, and followed by heteronuclear ^{13}C HSQC, ^{13}C NOESY-HMQC and ^{13}C HCCH-TOCSY spectra.

3.3.3.1. Assignments using homonuclear spectra

The nonexchangeable protons were assigned by experiments recorded on samples in 100% D_2O. The samples were subsequently lyophilized, exchanged at least twice with 99.8% D_2O to remove most of the exchangeable protons, and dissolved in 99.996% D_2O NMR buffer.

A variety of homonuclear spectra were recorded. Two-dimensional homonuclear NOESY experiments were acquired with mixing times of 50 ms, 100 ms, 150 ms and 300 ms to monitor NOE buildups. Two-dimensional DQF-COSY and TOCSY (11 ms and 50 ms) experiments were recorded to identify pyrimidine H5/H6 resonances, and qualitatively determine sugar H1'-H2' coupling constants. These spectra were acquired mostly on the Varian 600 spectrometer with 6600 Hz sweep widths in both dimensions with $2048(t_2) \times 512(t_1)$ complex data points. All spectra were processed with Lorentzian-to-Gaussian window transformation and zero-filling to give a final data set of $4096(t_2) \times 1024(t_1)$ data points.

The assignment strategy of the nonexchangeable proton resonances in the helical stem was based on the standard "NOE walk" procedure (Wüthrich, 1986). Briefly, in a standard A-form (or B form) helix, a base aromatic proton (purine H8 or pyrimidine H6) and the adjacent 5'-side ribose protons (H1', H2' and H3') on the same strand are in close proximity and give sequential peaks. Because of relatively better dispersion and a more down field-shift of the H1' resonances, resolved sequential NOE connectivities can be resolved from the ribose H1' to the base protons in NOESY spectra with long mixing times (> 250 ms). Furthermore, an adenine H2 proton is also close in space to the adjacent 3'-side ribose proton H1' on the same strand as well as the H1' on the opposite strand, 3' to this base. Because an A H2 proton can be easily identified from a NOESY spectrum acquired in H_2O through the strong NOE to imino protons of its paired U, it usually serves as a starting point for the nonexchangeable proton resonance assignments.

On the basis of the above assignment strategy, two A H2 protons (A7 and A52) that were assigned from the NOEs to the base paired U imino protons were used as starting points. In addition, the base protons of the pyrimidines (H6) were differentiated from that of the purine base protons (H8) on the basis of strong intra-nucleotide H5-H6 cross peaks in both NOESY and COSY spectrums. H6 proton chemical shifts of six cytosines, the three uracils in helical regions and the one cytosine in the tetraloop were identified in this manner prior to the sequential assignment. Figure 3.3.3 shows a region of the NOESY (300 ms mixing time) spectrum containing the aromatic (H8/H6/H2) to anomeric (H1'/H5) cross peaks. The sequential

connectivity H8/H6 to H1' within the lower stem (G5 to C9, G61 to G65), the upper stem (G13 to C17, G50 to C54) and the tetraloop (G18 to A21) are indicated.

Since there was significant overlap between 7.8 to 8.0 ppm in the above region, the assignments were verified from the NOESY spectrum acquired in H_2O. First, C H5 proton in a G-C base pair was identified from the two strong NOEs to its amino protons and a weak NOE (through spin diffusion) to the paired G imino proton. For example, the C17 H5 chemical shift was verified through the G50H1-C17NH$_2$-C17H5, a characteristic pattern of a Watson-Crick base pair. In addition to the H5 protons, the H1' proton assignments were verified through the sequential NOEs from the G imino protons to H1' on the same strand 3' to the G, and to the H1' of the ribose on the opposite strand 3' to this base pair (Heus & Pardi, 1991a). For example, C9 H1' and U63 H1' can be identified through the NOEs to G62 H1 (see Figure 3.3.1). Using the above homonuclear approach, sequential NOE walks in the anomeric-aromatic region were made at the lower stem, upper stem and tetraloop regions. The connectivities were not observed in the internal loop region (G10 to A12 and A55 to U60), consistent with the absence of NOEs of the exchangeable protons in this region. Tentative base-sugar connections were made for the three (G10, G11, and A55) out of nine nucleotides in this segment. No further identification was made because the base and ribose peaks were broad in this segment.

The most unusual feature among the identified base and ribose H1' protons of the free L30N RNA is the large upfield shift of the H1' proton of G50 (4.00 ppm) from the C17-G50 base pair that flanks the stable GCAA tetraloop. Such an upfield shift has been reported from earlier studies on GNRA hairpin loop-stem structures (Heus & Pardi, 1991b). Also an interesting upfield shift of a ribose proton (4.89 ppm) which show a strong NOE to an aromatic proton H8 (7.24 ppm) was observed within the internal loop. Due to the lack of sequential connectivity within the internal loop, the assignment was not made to these two chemical shifts.

After the sequential connections of the aromatic to H1' protons, an attempt next was made to correlate the H1' protons with the remaining sugar protons (H2', H3', H4' and H5', H5") using the conventional relay experiments (*i.e.*, COSY and TOCSY). These approaches are generally
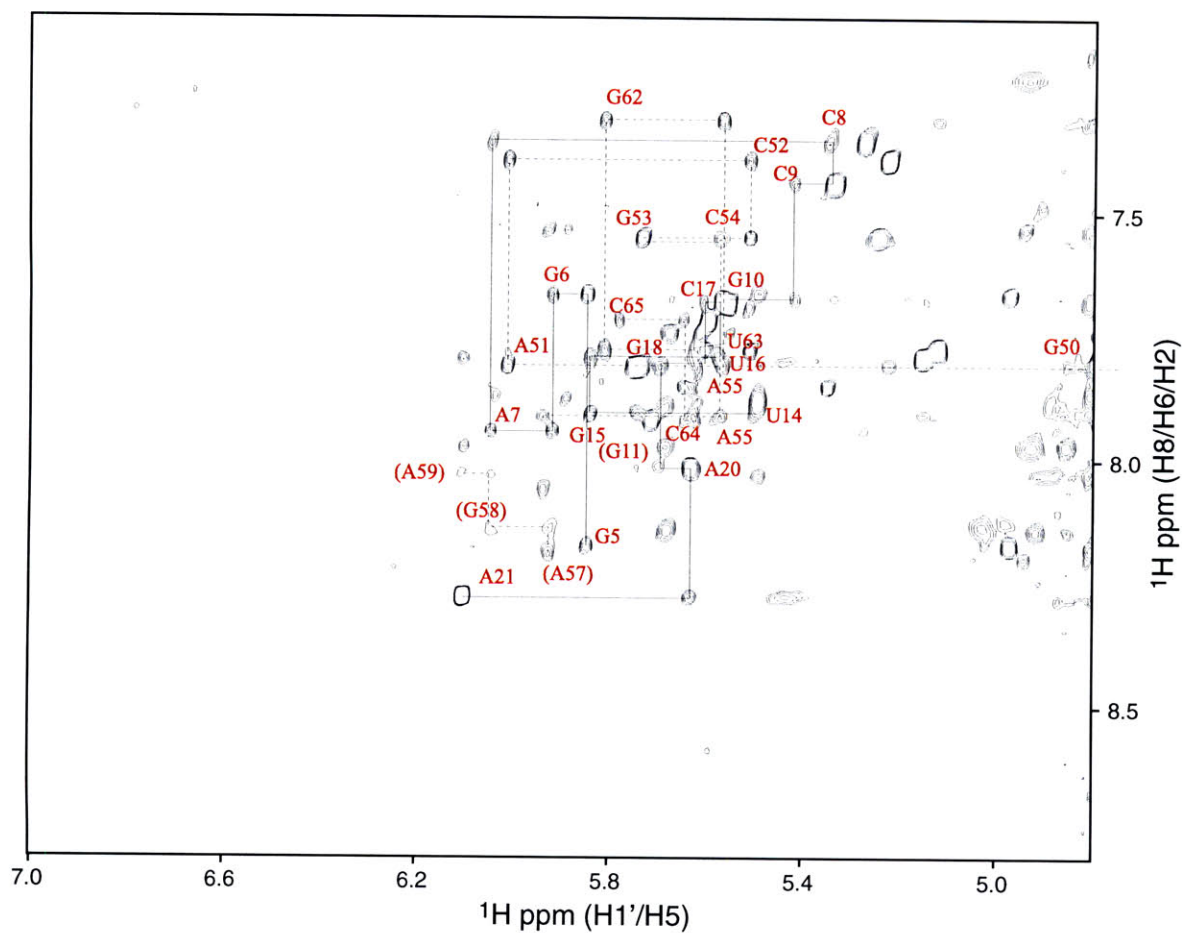
Figure 3.3.3: Aromatic (H8/H6/H2) to ribose H1' proton regions of a 250 ms NOESY spectrum of L30N RNA recorded at 25˚C. The solid lines trace the NOE sequential connections from G5 to G10 and U14 to A21, and dashed lines trace the connections from G50 to A55 and G61 to C65. The labels indicate intranucleotide H8/H6 to H1' NOEs. The G50 H1' resonance is shifted downfield at 3.95 ppm and is not included in this plot. Tentative assignments are indicated in parentheses.

difficult when applied to sugars protons in an A-form RNA because of the small H1'-H2'
coupling constant (1-2 Hz) for the C3'-endo sugar conformation. But these can be useful for
identifying ribose with unusual sugar conformations (*e.g.*, C2'-endo having *J*-coupling of up to
12 Hz). While most of the H1'-H2' correlations for those in C3'-endo conformation are missing
in either COSY or TOCSY spectra, sugars that adopted the unusual C2'-endo conformation are
easily identified in these experiments, because of the large H1'-H2' coupling constant (up to 12
Hz). H1'-H2' cross peaks of four residues (G5, G11, A20 and A21) were identified in COSY and
TOCSY (11 ms mixing time) experiments, suggesting possible C2'-endo conformations for these
nucleotides. H3' protons of these nucleotides were subsequently identified using a 100 ms
mixing time TOCSY experiment (H1'-H2'-H3' correlation). No further assignment was made for
the remaining ribose protons (H4', H5' and H5") as the TOCSY relays were very weak for these
protons. For the other nucleotides, almost no through-bond correlations could be made from H1'
to the other sugar protons by either COSY or TOCSY experiments. This was either because of
the small H1'-H2' coupling constants of sugars in the helical region, or because of conformational
broadening in the internal loop region. Not only the *J*-correlated assignments were impossible
for these nucleotides, a NOE-based through-space assignments were also not favorable. Despite
the fact that a short mixing time NOESY (50 ms) could potentially help to identify H2' or H3'
sugar protons, severe overlap of these resonances made assignment very difficult and less
reliable. The small homonuclear coupling constants and resonance overlaps limited the
application of the homonuclear experiment to identify the sugar resonances. Consequently, it
was necessary to resort to a ^{13}C-labeled RNA sample to complete the assignments.

3.3.2.2. Assignments using the heteronuclear spectra

The ^{13}C/^{15}N uniformly labeled L30N RNA were very useful for the assignments for the
nonexchangeable protons. Besides helping in resolving overlaps in the NOESY spectra by ^{13}C-
edition, it also helped in the TOCSY transfers using the large $^{1}J_{CC}$ coupling constants in the
HCCH-TOCSY experiments

Two types of isotopic labeling patterns were introduced in the RNA heteronuclear NMR experiments. These were the uniformly $^{13}C/^{15}N$-labeled and $^{13}C/^{15}N$-guanosine-only labeled L30N RNA. While the $^{13}C/^{15}N$-labeled sample was used to identify all ^1H, ^{13}C, ^{15}N resonances in the RNA, the $^{13}C/^{15}N$-guanosine only labeled sample was used to assist the purine assignments, particularly in the internal loop. The majority of the experiments were acquired on the Varian 600 MHz spectrometer; a few 3D experiments were acquired on the Varian 750 MHz spectrometer. Where appropriate, ^{13}C and ^{15}N decoupling during acquisition was accomplished with a GARP pulse train. Two-dimensional HSQC and various 3D ^{13}C-NOESY-HSQC and HCCH-TOCSY experiments were acquired to obtain RNA assignment. Three-dimensional NOESY and TOCSY experiments were recorded on the uniformly $^{13}C/^{15}N$-labeled sample to obtain RNA assignment in the free form. Using the sample in H_2O, 3D CN-NOESY-HSQC spectra were acquired centering the ^{15}N transmitter around 120 ppm to observe NOEs originated from all exchangeable proton, and ^{13}C around 104 ppm to observe NOEs from nonexchangeable aromatic and H1' protons. It was then exchanged into D_2O, and 3D ^{13}C-HSQC-NOESY spectrum of 150 ms mixing time and 3D HCCH-TOCSY of 12 ms mixing time was recorded, the ^{13}C transmitter was centered around 83 ppm for the sugar proton assignments. All 3D experiments were performed on the Varian 600 MHz spectrometer at 25°C.

Two-dimensional ^{13}C-HSQC spectra at 15, 25 and 30°C were acquired by either centering the ^{13}C transmitter at 116 ppm to maximize aromatic and H1'/C1' resonance ^1H-^{13}C correlations, or around 83 ppm to obtain anomeric ^1H-^{13}C correlations. Constant time ^{13}C-HSQC spectra, centered at 83 ppm were acquired at 25 and 30°C with evolution periods optimized for $J_{HC} = 200$ Hz, and the constant-time period set to $1/J_{CC}$ (about 27 ms). In the aromatic region (140-160 ppm), H8, H6 and H2 protons were identified by the chemical shift of the directly attached carbon. The adenine H2s are well resolved from the rest of aromatic protons as the C2 chemical shifts are more down field. Among them, four of eight adenine H2/C2s exhibited relatively strong correlation peaks, and the rest were broad and nearly unrecognizable. Based on the imino to H2 NOE pattern in a 3D CN-NOESY-HSQC experiment, these four H2/C2s were identified as those of the A20 and A21 from tetraloop (through the G18 imino) and of A7 and A51 from two helical stem regions (through the respective U63 and U16 imino protons). The other four AH2

protons within the internal loop regions were not identified as a result of exchange broadening. While A H2/C2 are resolved from the other aromatic resonances, the purine H8 and pyrimidine H6 resonances fall in a region with ^{13}C chemical shifts between 135-144 ppm. The H6/C6s of pyrimidines are distinguished from purine H8/C8 through the strong H6-H5 NOEs in the 3D NOESY-HSQC experiment. The differentiation of uracil and cytosine within the pyrimidines can be differentiated indirectly through two NMR observables. The C5 chemical shift of a cytosine has a more upfield shift than that of a uracil. In addition, a cytosine H5 also has strong of NOEs to its exocyclic amino protons observed in a 3D CN-NOESY-HSQC spectrum. All eight C's and three out of four U's were identified this way. It should be noted that the correlation cross peaks of H5/C5 and H6/C6 of U60 in the proposed G10-U60 base pair were not observed due to the broadening of the base pair that flanks the internal loop. The pyrimidine H6/C6 resonances were identified through characteristic intra-nucleotide peaks, whereas the unambiguous differentiation of purine H8/C8s are achieved through a $^{13}C/^{15}N$ guanine-only labeled sample. After excluding the correlation of G H8/C8 that are unambiguously identified in a ^{13}C-edited HSQC spectrum on the G-only labeled sample, and the remaining H8/C8 cross peaks in the fully labeled sample were identified as those from adenosines. Using a 3D CN-NOESY-HSQC experiment, specific assignment were made for ten G's and five A's from the thirteen G's and seven A's observed in the ^1H-^{13}C HSQC spectrum. The unassigned bases were mainly due to weak correlation and lack of NOE cross peaks for these resonances. Nevertheless, a guanine residue was unusually showed an upfield shift of H8 (7.24 ppm) and down field shift of C8 (140.8 ppm). Although this G is unassigned at the moment, it was to be in the internal loop location and special chemical shift features suggested this residue adopts an unusual conformation.

Similar to aromatic protons in the bases, the resolution of ribose protons were also spread out by the attached ^{13}C chemical shifts and can be observed in a simple ^{13}C-edited HSQC spectrum (Figure 3.3.4). Furthermore, in a ^{13}C-edited CT-HSQC spectrum (T = 27 ms), C1's and C5's not only were separated from C2', C3' and C4' by chemical shifts as those in a normal ^{13}C-HSQC spectrum, but they also displayed opposite signs from C2', C3' and C4' resonances. This special feature was particularly useful in unambiguous identifications (*e.g.*, the G50 C1' that is

close to C4' resonance chemical shifts, and the C65 C3' that is close to C5' resonances chemical shift).

With the ^{13}C chemical shifts from the HSQC spectrum and the H8, H1' proton resonance assignments made in homonuclear experiments, HCCH-TOCSY and NOESY-HMQC experiments were used to correlate the sugar protons through scalar coupling and through close proximity in space, respectively. Figure 3.3.5 shows several representative planes featuring the through bond correlation of the sugar protons in nucleotide C8 using HCCH-TOCSY experiment of uniformly ^{13}C/^{15}N-labeled RNA. Because of the excellent dispersion in both the ^{1}H and ^{13}C chemical shifts of H1' and C1', many of the H2's, H3's, H4's and even H5's/H5''s were correlated to the H1' in TOCSY experiment. In addition, the sequential NOEs from the sugar H2' and H3' to its own and adjacent 3' side aromatic H8/H6 protons, and NOEs from sugar H5', H5'' to its own H8/H6 protons, significant amount of assignments were obtained. Despite of the presence of overlap even in three-dimensional experiments, close to 90% sugar resonances were assigned in the helical stem region that are listed in Table 3.2. The general acquisition parameters used in all the homonuclear and heteronuclear experiments performed on the free L30 RNAs are listed in Table 3.3.

Almost complete assignments for all the riboses in both helical stems and the tetraloop were made through analysis of ^{13}C edited constant time (CT-) HSQC, NOESY-HMQC and HCCH-TOCSY spectra acquired in D_2O at 25°C. In summary, the NOE and sugar coupling constant patterns were consistent with the formation of standard A-form helices with C3'-endo sugar pucker for the lower and upper stems. The H2 protons for adenines at positions A7 and A51 in the stems exhibit directional NOEs to same-strand and cross-strand ribose H1' protons that are also characteristic of A-form helices in solution.

In summary, in order to study the L30 protein pre-mRNA binding site in NMR spectroscopy, an optimal L30N RNA was designed on the basis of biochemical analyses that include protein binding and size exclusion chromatography assays. The resonance assignments of the L30N RNA in the free form were carried out using a combination of homonuclear and
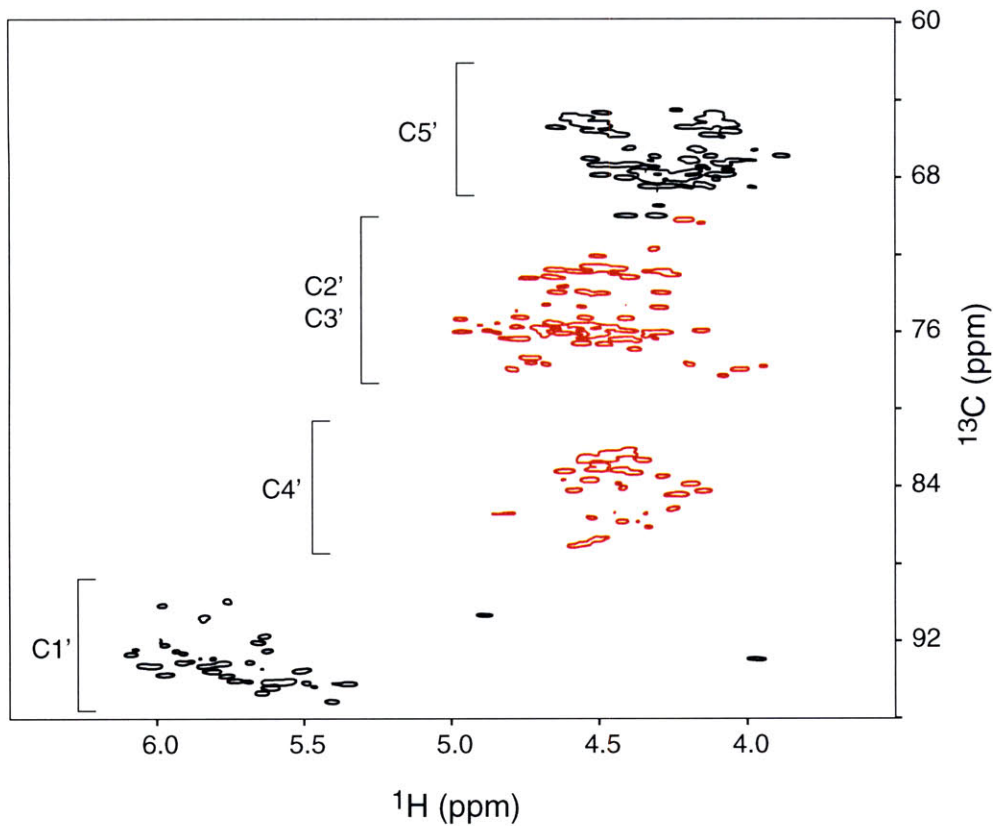
Figure 3.3.4: Ribose region of the ^{1}H-^{13}C CT-HSQC spectrum acquired on a uniformly ^{13}C/^{15}N-labeled L30N RNA at 25°C with T = 27 ms. Negative cross peaks (in red) correspond to ^{13}C nuclei (*i.e.*, C2', C3' and C4') that are coupled to two carbons. Positive cross peaks (in black) correspond to ^{13}C nuclei (*i.e.*, C1' and C5') that are coupled to one carbon. Characteristic chemical shift ranges are denoted in brackets.
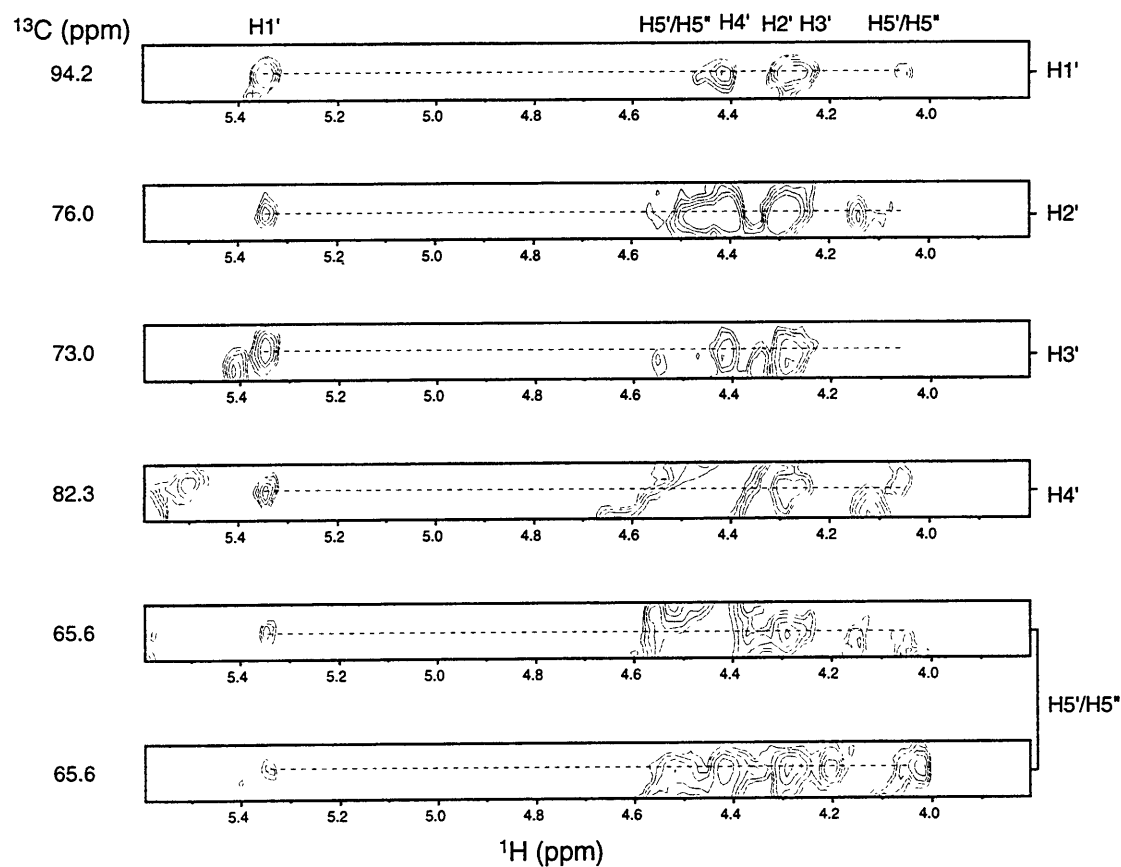
Figure 3.3.5: Selected slices of a 12 ms 3D HCCH-TOCSY spectrum of the ^{13}C/^{15}N-labeled L30N RNA at 25°C. These six regions of ^{13}C slices (chemical shifts are indicated on the left) represent the correlations originated from H1', H2', H3', H4' and H5'/H5" of residue C8.

Table3.2: Free form L30N RNA exchangeable and nonexchangeable proton, nitrogen and carbon chemical shifts at 25°C. Proton chemical shifts are referenced to internal TSP. Nitrogen and carbon chemical shifts are referenced to external NH₄Cl and TSP, respectively. The errors in chemical shifts are ± 0.02, 0.2, 0.3 ppm for proton, nitrogen and carbon, respectively. Tentative assignments are indicated in ().

Residue	H1,H3/N1,N3	amino/amN	H8,H6/C8,C6	H2,H5/C2,C5	H1'/C1'	H2'/C2'	H3'/C3'	H4'/C4'	H5',H5''/C5'
G5			8.18/139.4		5.85/90.8	4.97/75.5	4.75/75.4	4.58/84.2	4.42,4.33/67.8
G6	12.50/146.8		7.67/137.6		5.92/93.1	4.69/75.9	4.65/74.0	4.61/83.2	4.37,4.51/67.4
A7			7.94/140.5	7.86/154.4	6.05/93.3	4.63/76.1	4.63/73.1	4.55/82.5	4.20,4.65/65.4
C8		8.39.7.13/97.2	7.36/141.0	5.28/97.7	5.35/94.2	4.29/76.0	4.26/73.0	4.42/82.3	4.06,4.47//65.6
C9		8.35,6.67/97.1	7.44/141.2	5.34/98.2	5.42/95.1	4.55/75.2	4.29/72.8	4.35/82.6	4.07,4.52/65.2
G10	10.80/144.6		7.70/137.0		5.57/93.2	4.48/78.0	4.78/75.3	4.29/83.5	
G11	10.92/146.0		7.93/139.4		5.68/89.1	4.86/79.2	4.96/75.9	4.55/87.1	4.08
A12			8.14/142.8	8.00/154.9	5.99/93.8	4.15/75.9	4.52/73.0	4.63/82.2	
G13	12.96/147.4		8.06/138.3		5.99/92.3	4.90/75.5	4.32/70.5		
U14	11.93/158.5		7.89/141.4	5.49/104.45	5.49/94.3	4.46/75.9	4.42/72.7	4.39/82.29	4.26,4.32/67.9
G15	12.79/147.4		7.91/137.7		5.84/93.3	4.52/75.7	4.59/73.6	4.55/82.7	4.40,4.08/67.4
U16	14.36/162.4		7.80/142.3	5.15/103.0	5.58/94.5	4.51/76.0	4.43/73.1	4.44/82.3	4.15,4.54/65.3
C17		8.28,6.88/97.9	7.68/141.2	5.56/98.4	5.61/94.7	4.30/76.0	4.50/72.6	4.47/82.4	4.11,4.57/64.9
G18	10.74/146.0		7.70/137.0		5.62/92.4	4.56/76.7	4.68/74.9	4.43/83.2	4.43,4.12/65.9
C19		7.21/97.1	7.82/144.5	5.75/98.6	5.70/92.0	4.40/76.4	4.30/76.0	4.15/84.2	4.15,3.98/66.6
A20			7.21/97.1	7.90/155.0	5.63/91.7	4.37/77.5	4.57/76.6	4.22/84.4	3.77,3.88/66.9
A21			8.02/141.1	8.10/155.3	6.10/97.8	4.73/77.4	5.41/75.5	4.54/83.6	4.44,4.53/67.4
G22	12.35/146.6		8.28/142.8		4.00/92.9	4.42/75.4	4.30/74.8	4.40/83.3	4,41,4.31/70.1
A51			7.81/139.9	7.78/154.2	6.02/93.3	4.47/76.1	4.64/72.7	4.47/82.6	4.13,4.56/64.9
C52		8.45,6.95/98.3	7.40/141.0	5.22/97.8	5.51/93.6	4.41/76.4	4.40/73.1	4.41/82.1	4.07,4.51/65.3
G53	11.14/143.9	6.43/72.9	7.54/137.6		5.73/94.0	4.65/75.6	4.28/74.1	4.52/83.1	4.08,4.41/64.8
C54		8.10,7.13/98.8	7.54/140.5	5.25/97.7	5.57/94.2	4.47/75.7	4.52/72.0	4.47/82.5	4.11,4.52/64.8
A55			7.91/139.7		5.94/93.8	4.78/76.3			
G56	10.99/144.0		7.24/140.8		4.89/90.8	4.50/77.8	4.68/75.8	4.15	4.08,4.49/68.0
A57			8.21/141.6		6.04/89.3	4.91	4.7	4.58	4.31
G58			8.15/139.6		5.99/87.5	5.04/76.8	5.01/75.0	4.31	
A59			8.11/141.6		5.77/93.9	4.49/76.5	4.73/73.3		
U60	11.79/158.4		7.69/142.5	5.51	5.69/93.0				
G61	12.69/146.7		7.82/137.9		5.57/94.2	4.76/75.3	4.55/74.0	4.50/83.0	4.30/68.6
G62	13.34/148.2		7.31/136.7		5.81/93.6	4.53/75.8	4.56/72.9	4.51/82.6	4.54,4.11/65.1
U63	14.47/162.5		7.78/142.3	5.12/102.9	5.62/94.3	4.57/75.6	4.51/72.7	4.46/82.2	4.13,4.44/65.3
C64		8.64,7.13/98.2	7.92/142.3	5.72/98.1	5.64/94.3	4.40/76.8	4.68/74.5	4.48/83.0	4.15,4.52/64.6
C65			7.72/142.3	5.60/98.7	5.78/93.1	4.03/77.9	4.20/70.2	4.20/83.8	4.06,4.54/65.6

Table 3.3: Data acquisition parameters and pulse sequences used in free form L30 RNA NMR experiments. The four subdivisions in each NMR dimension (*F1*, *F2*, or *F3*) are: **a**, complex data points, **b**, spectral width (Hz), **c**, carrier position (ppm) and **d**, nuclei. In a 2D experiment, *F2* is the direct acquisition dimension, whereas *F1* is the indirect detection dimension. In a 3D experiment, *F3* is the direct acquisition dimension, whereas *F1* and F2 are the indirect detection dimensions.

Molecule	Experiment	*F3*(a, b, c, d)	*F2*(a, b, c, d)	*F1*(a, b, c, d)	T°C	Mixing time (ms)	Reference
L32N RNA	NOESY (Bitter 501)	-	4096, 5500.6, 4.87, ^{1}H	512, 5500.6, 4.87, ^{1}H	15°C	50, 100, 200, 400	
	NOESY (Varian 600)	-	2048, 6000, 4.78, ^{1}H	512, 6000, 4.78, ^{1}H	25°C	300	
	NOESY-watergate (Bitter 501)	-	4096, 11990 4.91, ^{1}H	512, 11990, 4.91, ^{1}H	5, 15, 25°C	100, 150	
	dq-COSY (Varain 600)	-	2048, 6000, 4.78, ^{1}H	512, 6000, 4.78, ^{1}H	25°C		(Rance *et al.*, 1983)
	TOCSY (Bitter 501)	-	2048, 5500.6, 4.87, ^{1}H	512, 5500.6, 4.87, ^{1}H	15°C	75	(Brauschweiler & Ernst, 1983)
L32(A$_{57}$C) RNA mutant	NOESY (Varian 600)	-	2048, 6000, 4.78, ^{1}H	512, 6000, 4.78, ^{1}H	15, 25°C	300	
	dq-COSY (Varain 600)	-	2048, 6000, 4.78, ^{1}H	512, 6000, 4.78, ^{1}H	15, 25°C		(Rance *et al.*, 1983)
L32(U$_{14}$C) RNA mutant	NOESY-watergate (Varian 600)	-	2048, 14000, 4.91, ^{1}H	512, 14000, 4.91, ^{1}H	10°C	150	
	dq-COSY (Varain 600)	-	2048, 6000, 4.78, ^{1}H	512, 6000, 4.78, ^{1}H	15, 25°C		(Rance *et al.*, 1983)
L32(A$_{55}$C) RNA mutant	NOESY-watergate (Varian 600)	-	2048, 14000, 4.87, ^{1}H	512, 14000, 4.87, ^{1}H	15°C	100	

L32(A$_{55}$U) RNA mutant	NOESY-watergate (Varian 600)	-	2048, 14000, 4.87, ^{1}H	512, 14000, 4.87, ^{1}H	15°C	100	
^{15}N,^{13}C Labeled L32N RNA	^{15}N FHSQC (Bitter 501)	-	1024pts 11000, 4.87, ^{1}H	128, 5000.0, 117.91, ^{15}N	15°C		(Mori *et al.*, 1995)
	^{13}C FHSQC (Varian 600)	-	1024, 7000, 4.87, ^{1}H	128, 14000Hz 116.17, ^{13}C	15, 25, 30°C		(Mori *et al.*, 1995)
	^{13}C HSQC (Varian 600)	-	1024, 6000, 4.78, ^{1}H	128, 7000, 82.97, ^{13}C	25°C		(Bax *et al.*, 1990)
	^{13}C HSQC-CT (Varian 600)	-	1024, 6000, 4.78, ^{1}H	200, 7000, 82.97ppm ^{13}C	25°C		(Santoro & King, 1992)
	^{13}C HSQC-NOESY (Varian 600)	512, 6000, 4.78, ^{1}H	128, 6000, 4.78, ^{1}H	32, 4000, 82.97, ^{13}C		150	(Clore *et al.*, 1990)
	^{13}C HCCH-TOCSY (Varain 600)	512, 5000.0, 4.78 , ^{1}H	128, 3000.0, 4.78 , ^{1}H	32, 4000, 82.97, ^{13}C	25°C	12	(Clore *et al.*, 1990)
	^{15}N,^{13}C HSQC-NOESY (Varian 600)	512, 6600, 9.00, ^{1}H	96, 6600, 9.00, ^{1}H	25, 4000, 119.58, ^{13}C (^{15}N)		150	(Pascal *et al.*, 1994)
^{15}N,^{13}C-Guanosine Labeled L32N RNA	^{15}N FHSQC (Bitter 501)	-	1024, 11000, 4.91, ^{1}H	128, 5000.0, 117.91, ^{15}N	15°C		(Mori *et al.*, 1995)
	^{13}C FHSQC (Varian 600)	-	1024, 7000, 4.87, ^{1}H	128, 14000Hz 116.17, ^{13}C	15, 25, 30°C		(Mori *et al.*, 1995)
	^{13}C HSQC (Varian 600)	-	1024, 6000, 4.78, ^{1}H	128, 7000, 82.97, ^{13}C	25°C		(Bax *et al.*, 1990)
	^{13}C HSQC-CT (Varian 600)	-	1024, 6000, 4.78, ^{1}H	200, 7000, 82.97, ^{13}C	25°C		(Santoro & King, 1992)

heteronuclear experiments. The assignment strategy involves sequential NOE through-space approach to identify base pairings and sequential aromatic-sugar connection, and TOCSY through bond relay to correlate the intraresidue sugar proton resonances. Ninety-five percent of the proton resonances in the two helices and the tetraloop region, and few in the internal loop region were assigned using the above approaches. Based on the observable NOE patterns, the L30N RNA has been characterized as an undefined internal loop flanked by two helical stems in the absence of its cognate protein.

CHAPTER 4. The L30 Protein in the Bound Form

Though the structure of the protein-RNA complex is the ultimate goal in understanding the mode of recognition, it was very helpful to study the protein and the RNA in the free form. Most importantly, the studies on the free forms help in defining the changes that the protein and the RNA undergo upon complex formation and hence give clues to the mechanism. In addition, with respect to the NMR experiments, the studies on the free forms also help in analyzing the spectra of the complex. In a complex, due to the increased molecular weight, the correlation times become long, and as a consequence, the peaks broaden out. On the other hand, the protein and/or RNA NMR changes in the free and complex forms are generally predictable. The analyses of the free forms provide a solid foundation for determining the structure of the complex. Thus, with the data from the free L30 protein and RNA as described in the previous chapters, the studies on the L30 protein-RNA complex were initiated.

4.1. Complex Formation

4.1.1. RNase activity assay

Presence of any trace amount of RNase in the sample can lead to RNA degradation. Hence prior to formation of the L30 protein-RNA complex, it was necessary to conduct an RNase activity assay to ensure the quality of the L30 protein. Since the protein studied in the RNA complex was purified through native column purification, trace amounts of RNases are likely to co-purify with the MBP-L30 fusion and/or L30 proteins. The contamination of RNases, even a very small amount, can lead to significant degradation of the RNA within the complex. RNA degradation can be easily spotted in a NOESY spectrum by the presence of cross peaks having opposite signs to the diagonal, which derives from small molecules (*i.e.*, pyrimidine nucleotides, small oligos) that have been degraded from the RNA. The same was observed for the L30N RNA. Furthermore, the presence of degradation products was later confirmed by autoradiography by incubating trace amounts of [α-^{32}P] body labeled L30N RNA with the NMR

sample of the complex overnight. The addition of an RNase inhibitor in the millimolar RNA sample did not seem to help in reducing the degradation during NMR experiment. It was further found that the lack of a reducing agent (*e.g.*, DTT) in the solution increased the release of the previously bound RNases from the RNase inhibitor and actually accelerated the degradation.

Under such circumstances, it is best if the RNases can be removed from the protein earlier in the purification steps. The major contamination appeared to copurify on the CM column. Since the CM column non-selectively binds net cationic proteins, RNases with mostly positive charges could co-purify with MBP-L30 and/or L30 protein. Thus, affinity-based amylose column purification was used to remove the RNases from MBP-L30 protein. To be sure of the purification, L30 protein was always subjected to RNase activity assays prior to complex formation. Briefly, millimolar quantities of L30 protein were incubated with an equivalent amount of cold L30N and trace amounts of $[\alpha\text{-}^{32}P]$ body labeled L30N RNA in the NMR buffer at 37°C over a period of a week. Aliquots were taken during the course of incubation and were subjected to 20% PAGE gel analysis. The RNA degradation products generated from the contaminants in the L30 protein were compared to those in the absence of protein. Less than 10% overall degradation after a week was considered reasonable for proceeding with NMR measurements of the complex.

4.1.2. Titration

For the complex formation, it is necessary to have accurate concentrations of the individual protein and RNA. The concentration of L30 protein was determined based on the UV absorbance at 274 nm which derives from its six tyrosine residues (each Tyr has an average extinction coefficient $\varepsilon_{274.6\,nm}$ = 1420 M^{-1} cm^{-1}) (Creighton, 1984). L30 protein has the same absorbance (at 274 nm) under native (10 mM sodium phosphate, pH 7.0) and denaturing conditions (8M Urea or 6M guanidine hydrochloride), with less than 5% difference. Therefore, the protein concentration was quantified under the native condition after dialysis in the phosphate buffer. But the RNA, containing both single-stranded and double-stranded regions, has an

extinction coefficient that is not easy to predict in its native fold. Therefore the RNA was subjected to overnight alkaline hydrolysis to generate individual nucleotides which give more accurate measurements using UV absorbance (the sum of the individual extinction coefficients for the nucleotides is $\varepsilon_{260\,nm} = 363$ mM^{-1} cm^{-1}).

Upon accurate determination of both protein and RNA concentrations, the complex was formed by titration. Unlike the typical titration performed in the NMR tube for most complexes, direct addition of either protein or RNA into its counter part at millimolar concentration leads to severe, irreversible aggregation. Thus an alternative method was involved in which the protein and the RNA at 1:1 stoichiometry were gently mixed in batches in a 1.5 ml Eppendorf tube at a concentration of 50 μM, and these were transferred to a 2 ml Centricon-3 (Amicon) microconcentrator tube. The volume (almost 20 ml) was gradually reduced to about 500 μl by centrifugation of the Centricon tube at 6,000 g. The concentrated complex was then dialyzed against one liter of NMR buffer (10 mM potassium phosphate, pH 5.5 - 6.5, 0.02% NaN$_3$ and 0.1 mM EDTA) over 24 hrs with several changes of buffers.

4.2. NMR Characterization of the L30 Protein in the Bound Form

4.2.1. Optimization of the NMR sample condition

Similar to studies of the protein in the free form, optimization of NMR conditions (salt, temperature and pH) was necessary. ^{1}H-^{15}N-HSQC spectra of uniformly ^{15}N-labeled protein in complex with unlabeled RNA indicated that the protein demonstrates the best ^{1}H and/or ^{15}N linewidths and dispersion under low salt, pH of 5.5 in 10 mM potassium phosphate, and at elevated temperature. Therefore, subsequent NMR experiments on the protein in the complex were acquired at 30°C with the above buffer containing 0.02% NaN$_3$ and 0.1 mM EDTA.

In contrast to the free form of the protein, the protein in the complex is highly stabilized by the presence of L30N RNA and was not significantly affected by temperature and concentration changes. The CD thermal melt data indicated that the complex has a thermal denaturation transition around 68°C. Furthermore, up to a 2 mM NMR sample of the L30 complex remained stable at pH ranges from 5.5 to 6.5 at a temperature of 30°C for many weeks. The increased stability of the L30 protein in the presence of RNA was quite advantageous for the NMR studies. When experiments were recorded at elevated temperature, the spectra showed sharper linewidths and therefore better signal-to-noise ratios. Also, it was possible to record various triple resonance experiments as the sample had a prolonged lifetime, which were essential for complete ^{1}H, ^{15}N, ^{13}C$_\alpha$ and ^{13}C' assignments. A step-by-step description of the NMR assignments of the protein in the complex is given in the following subsections.

4.2.2. The sequential backbone assignments of the protein

4.2.2.1. General characterization of the backbone amides

The complex formation is, in general, reflected by the changes in the chemical shifts in the spectra. The most noticeable differences were observed in the amide proton chemical shifts. As shown in a ^{1}H-^{15}N HSQC spectrum acquired at 30°C (Figure 4.2.1), there is an increased dispersion in the chemical shifts upon complex formation. The peaks now span from 5.95 to 9.92 ppm as compared to 6.5 to 9.3 ppm for the free form of the protein. Furthermore, all of the 101 backbone amides, 8 pairs of side chain amides, and 3 arginine side chain amines were observed using ^{1}H-^{15}N HSQC spectra acquired at different temperatures and pHs. In addition, three distinct pairs of arginine guanidinium side chains were also observed (ranging from 6.55 to 7.86 ppm for ^{1}H and from 62 to 75 ppm for ^{15}N). This is an indication of hydrogen bond formation. Besides changes for some residues, the overall NMR spectra did not show very dramatic changes. Careful analysis of the HSQC spectra of the free and bound forms indicates that the most dramatic changes are for the peaks with either weak intensities or the peaks that are

absent in the free form. This suggests that sufficient stabilization occurs in some local conformation upon RNA binding.

4.2.2.2. NOE-based sequential assignments

NOE-based sequential assignment in the complex is more cumbersome as the additional RNA aromatic and sugar resonances interfere with the amide and H_α regions of the protein. Nevertheless, as the ^1H-^{15}N HSQC spectra of the free and complex protein are similar, an attempt towards NOESY-HSQC and TOCSY-HSQC based sequential assignments was made.

The ^{15}N-edited NOESY-HSQC and ^{15}N-edited TOCSY-HSQC spectra were acquire at the Varian 600 MHz spectrometer (unless noted otherwise). The amide proton resolved spectra were further analyzed using assignment procedures described in Chapter 2, section 2.2.2.1, on the basis of characteristic backbone sequential NOE patterns. Comparisons of the free and bound spectra were made at various stages to help in the assignment. While the chemical shifts for some residues vary between free and bound, a majority of the residues in either α-helix or β-sheet display similar sequential NOE patterns in the bound and in the free form. The 3D ^{15}N-edited NOESY-HSQC spectrum of the complex was much more sensitive than that of the free form, most probably due to the increased concentration and elevated temperature. As can be seen in Figure 4.2.2, which displays selected strips of backbone NOEs of residues L51 to A60 derived from a ^{15}N-edited NOESY-HSQC experiment acquired on a 750 MHz spectrometer, many more medium to long range NOEs were observed in this experiment. While the protein NOESY spectrum displays noticeable differences, the signal-to-noise ratio in the ^{15}N-edited TOCSY-HSQC experiment acquired on the complex did not show significant improvement as compared to the free form, and most of the relays from ^1H_N disappeared beyond H_βs in the ^{15}N-edited TOCSY spectrum. This is expected, as the NOE buildup rates increase with increased correlation time upon complex formation, whereas the signals in the TOCSY experiment decay much faster upon complex formation.
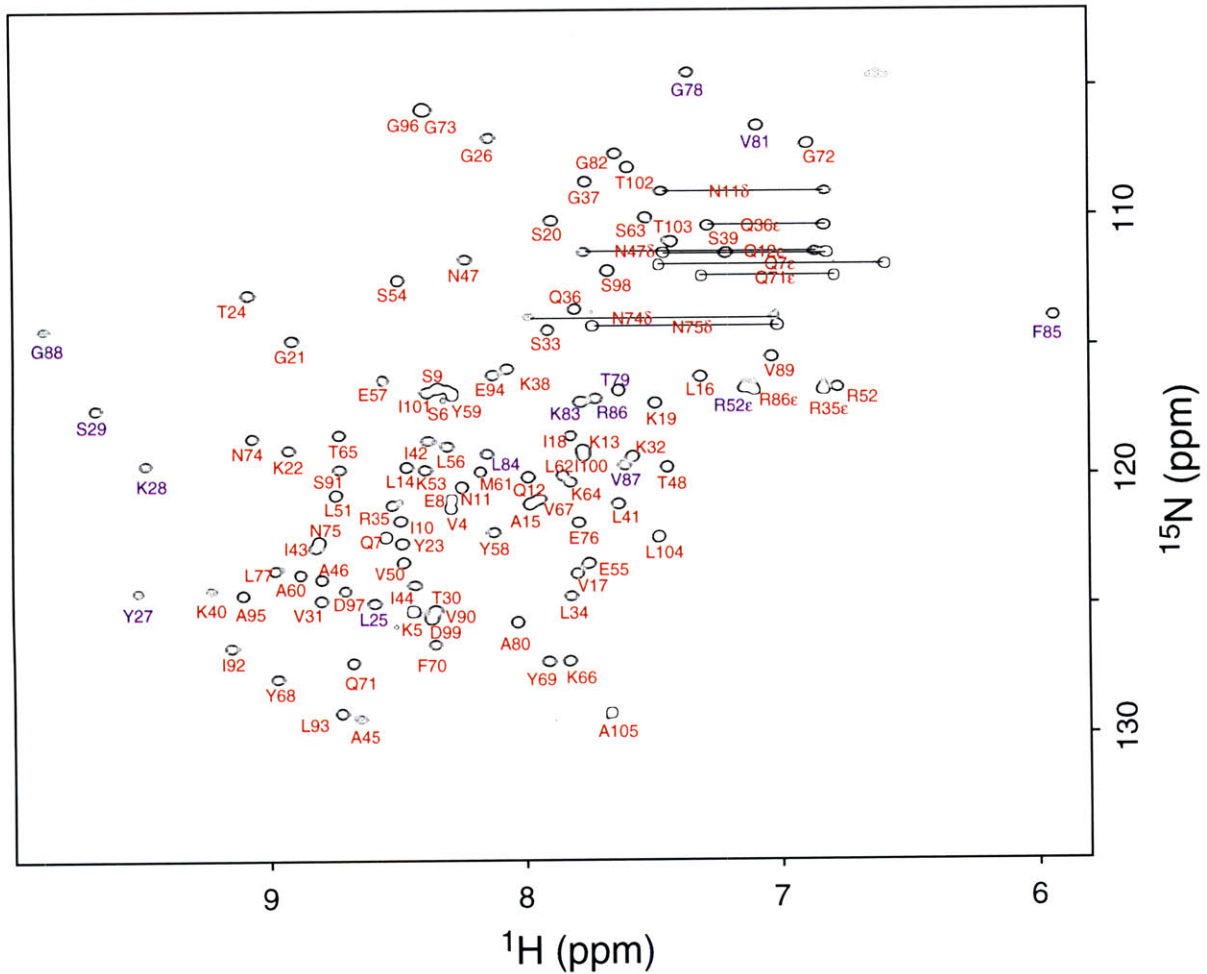
Figure 4.2.1: A ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled L30 protein in complex with L30N RNA at 30°C. The peaks are labeled with the assignments, with the one-letter code for the amino acid type followed by the sequence position. The side chain amides for Asn and Gln are connected with horizontal lines and labeled with the assignments with side chain positions in Greek letters. Arg ε side chain peaks are folded in this spectrum. Residues that undergo dramatic chemical shift changes upon RNA binding are indicated in purple, while others are indicated in red.
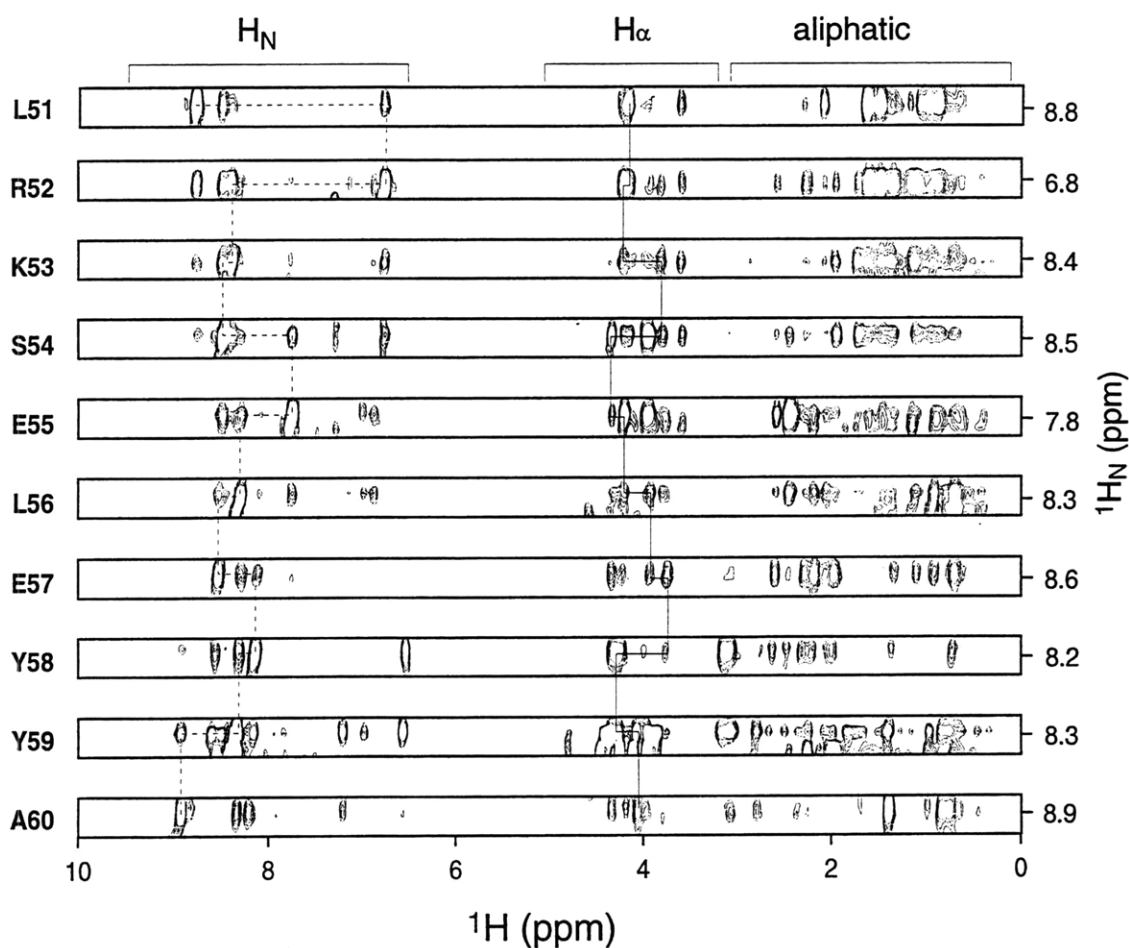
Figure 4.2.2: Representative NOESY strips from a ^{15}N NOESY-HSQC 3D spectrum of a ^{15}N-labeled L30 protein in complex with the L30N RNA. The sequential α-helical H_N-H_N (i, i+1) and H_α-H_N (i, i+1) NOE connectivities from residues L51 to A60 are highlighted with dashed lines and solid lines, respectively. Many long-range NOEs to aliphatic side chains are also observed in this spectrum.

Upon completion of the analyses on the NOESY and TOCSY spectra, it was noticed that most of the secondary structure present in the free protein is preserved in the bound form. However, new secondary structures were observed in the bound protein. Particularly, residues 74 to 87 in the bound form show noticeable sharpening of resonance linewidths. Not only have all of the backbone amide resonances been observed in the ^{1}H-^{15}N-HSQC spectra, they also display many medium-to-long range NOEs that are absent in the free protein. Based on characteristic NOE patterns, the segment from residues 76 to 81 was identified to adopt a helical conformation, while other residues are in a coil or loose turn conformation. Moreover, backbone amides of residues 27 to 29, 85 and 88 show dramatic chemical shift changes, some residues (27 to 29 and 88) with downfield shifts, and the other (85) an upfield shift. These changes indicate that these residues may be involved in binding the RNA.

4.2.2.3. Assignments based on triple resonance experiments

Though sequential backbone assignments of L30 protein in the complex were accomplished using a classical NOE-based approach, and with direct comparisons to that in the free form, there were doubts regarding assignments for residues with unusual NOEs or adopting unusual secondary structures (*e.g.*, tight turns). Missing or overabundant amide-amide NOEs further complicated the assignment process, and the presence of RNA aromatic and ribose resonances introduces additional confusion.

In order to corroborate the assignments, triple resonance experiments were recorded to provide complete backbone ^{1}H, ^{13}C and ^{15}N assignments. The basic building block of a triple resonance experiment consists of a set of pulses and delays during which magnetization is transferred between heteronuclei/homonuclei via large heteronuclei one- or two-bond scalar couplings. The experiments are named according to the spins that are frequency-labeled using HN, N, HA, CA, CO, and CB to represent the ^{1}H$_N$, ^{15}N, ^{1}H^a, ^{13}C$_\alpha$, ^{13}CO, ^{1}H$_\beta$, and ^{13}C$_\beta$ spins, respectively. These experiments share a common feature that is a so-called "out and back" pulse sequence in which the initially excited proton spin and the detected proton spin are identical. The sequential assignments based on these experiments are structure independent, as they

employ one- or two-bond *J* correlation for transfer, and hence provide an alternative and more reliable method than the NOE-based assignment.

There are two technical aspects that facilitate application of the triple resonance experiments to the L30 protein in the complex. First, in contrast to the protein in the free form, L30 protein in the complex is stable at low salt (10 mM potassium phosphate), which reduces the heating of the sample by extensive decoupling during magnetization transfer. Second, the protein is also stable at higher temperature (>30°C), therefore experiments recorded at the elevated temperature have sharper resonance linewidths. In addition to the technical reasons, there is also a practical necessity for using triple resonance on the complex. The complication of the protein spectra by the many RNA resonances at amide and α proton regions in the NOE base approach can be avoided by the through bond connection in triple resonance experiments. The approach used in triple resonance based assignment exploits the relatively large one-bond *J* couplings between the backbone ^{13}C and ^{15}N nuclei and between the backbone protons and the ^{15}N and ^{13}C$_\alpha$ nuclei to which they are directly attached. Therefore, correlation made by exploiting through bond scalar coupling in triple resonance experiments are more reliable than NOE-based through space connectivities.

Various triple resonance experiments were acquired on 0.75 mM ^{13}C/^{15}N doubly-labeled L30 protein in complex with unlabeled L30N RNA. These included HNCA (Ikura *et al.*, 1990), HN(CO)CA (Yamazaki *et al.*, 1994), HNCO (Muhandiram & Kay, 1994; Kay *et al.*, 1992) and HNCACB (Muhandiram & Kay, 1994). In all these experiments, WATERGATE was used at the end of the sequences for water suppression. Data for the above 3D experiments were processed using NMRPipe (Delaglio *et al.*, 1995). While processing the acquisition dimension (F3), a solvent suppression filter was applied to the data to minimize the water signal prior to apodization with a Lorentzian-to-Gaussian window function. Only the downfield half of the spectrum was retained in the 3D data set corresponding to the amide proton resonances. Small baseline distortions in F3 were removed by a first-order polynomial baseline correction. The second and third dimensions were processed subsequently by mirror image linear prediction (Zhu & Bax, 1990), apodized with a cosine-bell window function, and zero filled to double the

complex points. They were then Fourier transformed, phased, and with elimination of the imaginary half of the signal. In the case of the HNCACB experiment, the first data point was linear predicted to correct for the DC offset since the first point at t = 0 was not acquired. Peak positions and intensities for non-overlapping peaks were located interactively in the CAPP and PIPP (Garrett *et al.*, 1991) programs.

First, an HNCA (Ikura *et al.*, 1990) spectrum was recorded to correlate the amide ^{1}H and ^{15}N resonance with the ^{13}C$_{\alpha}$, which was important later in the HCCH-TOCSY correlation with the side chain resonances. This experiment correlates the amide ^{1}H and ^{15}N chemical shifts with the intraresidue ^{13}C$_{\alpha}$ shift, by making use of the relatively small one-bond ^{15}N-^{13}C J-coupling (7-11 Hz) to establish J correlation between the ^{15}N and ^{13}C$_{\alpha}$ spins. In addition, this experiment also provides sequential connectivities by transferring coherence from the ^{15}N spins to the ^{13}C$_{\alpha}$ of the preceding residue via the interresidue two-bond ^{15}N-^{13}C J-coupling (4-9 Hz). Thus for this experiment, the carbon carrier is set to 61.7 ppm in the C$_{\alpha}$ region, and the nitrogen carrier is set to 119.8 ppm in the backbone amide region. Magnetization originating on amide protons is transferred to the directly attached ^{15}N spin via an INEPT (insensitive nuclei enhancement by polarization transfer) sequence (Morris & Freeman, 1979). The ^{15}N chemical-shift is evolved during the t_1 period. Following t_1 evolution, the ^{15}N magnetization is transferred to the coupled ^{13}C$_{\alpha}$ spins using the delay period δ set for $1/2J_{N-C\alpha}$ coupling. During the subsequent t_2 evolution period, both ^{1}H and ^{15}N chemical shifts are refocused, and coherence evolution depends only on the ^{13}C$_{\alpha}$ chemical shift. The magnetization present following the t_2 evolution period is transferred back to the observable ^{1}H$_N$ magnetization by an INEPT transfer pathway. The HNCA experiment was acquired on a Varian Inova 750 MHz spectrometer. During the evolution and magnetization transfer periods, the carbonyls were decoupled using a shaped cosine-modulated WALTZ-16 pulse employed in a SEDUCE-1 profile (McCoy & Mueller, 1992), while proton decoupling is accomplished through WALTZ-16. All carbon pulses were applied using only a single carbon channel. The ^{15}N resonances were decoupled by a GARP sequence (Shaka *et al.*, 1985) during the acquisition period.

In addition to providing $^{13}C_\alpha$ assignments, the HNCA experiment also provided connectivities for the amide ^{1}H and ^{15}N to the C_α of the preceding residue, transferring coherence via the $^2J_{N-C\alpha}$ coupling. Since the magnitude of $^1J_{N-C\alpha}$ is usually larger than $^2J_{N-C\alpha}$ coupling, it was possible to distinguish C_α of intra- from interresidues, and to make connectivity through the peptide backbone. Figure 4.2.3 shows representative ^{15}N slices of the HNCA spectrum, from which sequential connectivities from residues 51 to 60 are made by the presence of stronger intra-residue C_α and weaker preceding residue C_α cross peaks in each H_N resonance. While most of the through-bond ^{1}H, ^{15}N and $^{13}C_\alpha$ alignments were made through this method, a few ambiguities in some assignments remained. For example, there were circumstances where $^1J_{N-C\alpha}$ and $^2J_{N-C\alpha}$ were comparable, which leads to two comparable cross peaks, and distinction between the intra- and interresidue $^{13}C_\alpha$ was not straightforward from only comparing intensities. There were also occasions where only one cross peak was observed, possibly due to degeneracy in adjacent residue $^{13}C_\alpha$ chemical shifts, or the absence of interresidue $^{13}C_\alpha$ correlation.

These ambiguities were resolved in a HN(CO)CA (Yamazaki *et al.*, 1994) experiment. While the HNCA experiment provides both intra- and inter-residue correlations, the HN(CO)CA experiment renders exclusively interresidue connectivities. Similar to the HNCA, an HN(CO)CA experiment transfers magnetization originating on an H_N proton to its directly bonded ^{15}N spin via an INEPT sequence, followed by the evolution of ^{15}N chemical shifts during the t_1 period. The difference is that in the subsequent step, the magnetization is transferred to ^{13}C' (carbonyl) through ^{15}N-^{13}C' coupling, and then to $^{13}C_\alpha$ through the ^{13}C'-$^{13}C_\alpha$ coupling, which establishes four-spin ^1H_N-^{15}N-(^{13}C')-$^{13}C_\alpha$ coherence. During the subsequent t_2 period, only the $^{13}C_\alpha$ chemical shifts are evolved and the ^{1}H and ^{15}N chemical shifts are refocused by applying 180° pulses on both at the midpoint of the t_2 period. Magnetization is then transferred back to the H_N protons for detection. Thus in the 3D spectrum, each peak is labeled by ^{15}N, $^{13}C_\alpha$, and H_N chemical shifts in the F1, F2, and F3 dimensions, respectively. The HN(CO)CA experiment was acquired on the Varian Inova 600 MHz spectrometer. While the ^{1}H and ^{15}N carriers were centered at 4.7 ppm and 118 ppm, respectively, the ^{13}C carrier was shifted during the experiment, jumping from 176 ppm for ^{13}C' to 58 ppm for $^{13}C_\alpha$. More specifically, the carbon carrier was centered at 176 ppm during carbonyl 180° pulses using a SEDUCE-1 (McCoy & Mueller, 1992)
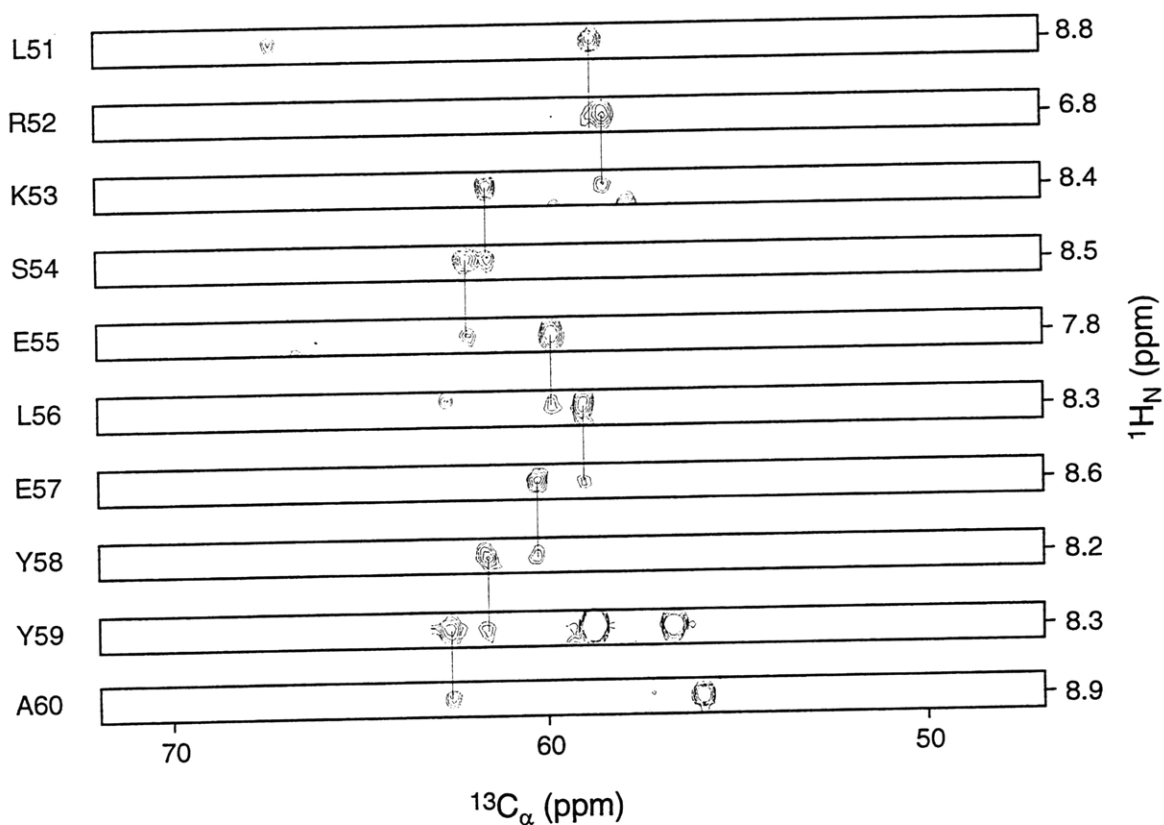
Figure 4.2.3: Representative strips showing the sequential C_α connectivities of residues L51 to A60 in the 3D HNCA spectrum of $^{13}C/^{15}N$-labeled, bound form L30 protein in H_2O. In each slice, the stronger cross peak corresponds to an intraresidue $H_{N(i)}$-$C_{\alpha(i)}$ correlation, and the weaker one corresponds to an interresidue $H_{N(i)}$-$C_{\alpha(i-1)}$ correlation. Sequential connectivities can be made in this manner.

profile with a peak amplitude of 4.72 kHz, and the carrier was then jumped to 59 ppm during the C_α period. $^{13}C_\alpha$ and $^{13}C_\beta$ off-resonance decoupling was achieved by a 400 μs REBURP (Geen & Freeman, 1991) pulse with a peak amplitude of 15.7 kHz, centered around 176 ppm. Decoupling of ^{15}N during acquisition was achieved through WALTZ-16. Figure 4.2.4 shows the same ^{15}N planes (as compared to the HNCA experiment) of residues L51 to A60 in the HN(CO)CA spectrum. The only cross peak observed corresponding to each amide proton was to the $^{13}C_\alpha$ of the preceding residue. Following this path, each C_α of the preceding residue is unambiguously assigned in case of the degeneracy in the HNCA experiment. In combination with both HNCA, HN(CO)CA experiments, all backbone sequential assignments were made.

The backbone sequential alignment made with the triple resonance experiment was subsequently compared to that made t in the NOE-based approach. While most of the backbone alignments showed consistency with the two independent approaches, two small segments with misalignments were identified. One was the reversal of backbone assignments for Gly72 and Gly73. The ^{15}N-edited TOCSY-HSQC did not allow differentiation between two glycine residues, thus the interresidue backbone H_N-H_N NOEs in the NOESY-HSQC spectrum were similar for both residues leading to the ambiguity. The other errors in the NOE-based sequential assignments were the misassignments of residues 81 and 83. Both showed strong NOEs to residue 82, and weak NOEs to the adjacent residues 80 and 84, respectively. Though the amino acid side chains were different (Val81 and Lys83), the peaks in the ^{15}N-edited TOCSY-HSQC were broad enough that incomplete transfer was observed. These two examples clearly support the power of using both triple resonance experiments as well as a different approach in checking the consistency of resonance assignments.

Despite the successes of both HNCA and HN(CO)CA experiments, attempts at an HNCACB experiment was unsuccessful. The HNCACB experiment correlates the amide ^1H and ^{15}N resonances with the $^{13}C_\alpha$ and $^{13}C_\beta$ resonances of the same and preceding residues via $^1J_{C\alpha N}$ and $^2J_{C\alpha N}$. The general magnetization transfer scheme is similar to that of an HNCA experiment with an extra delay for the transfer of the C_α magnetization to the C_β and back. The HNCACB experiment was performed on a Varian Inova 600 MHz spectrometer. All the experimental
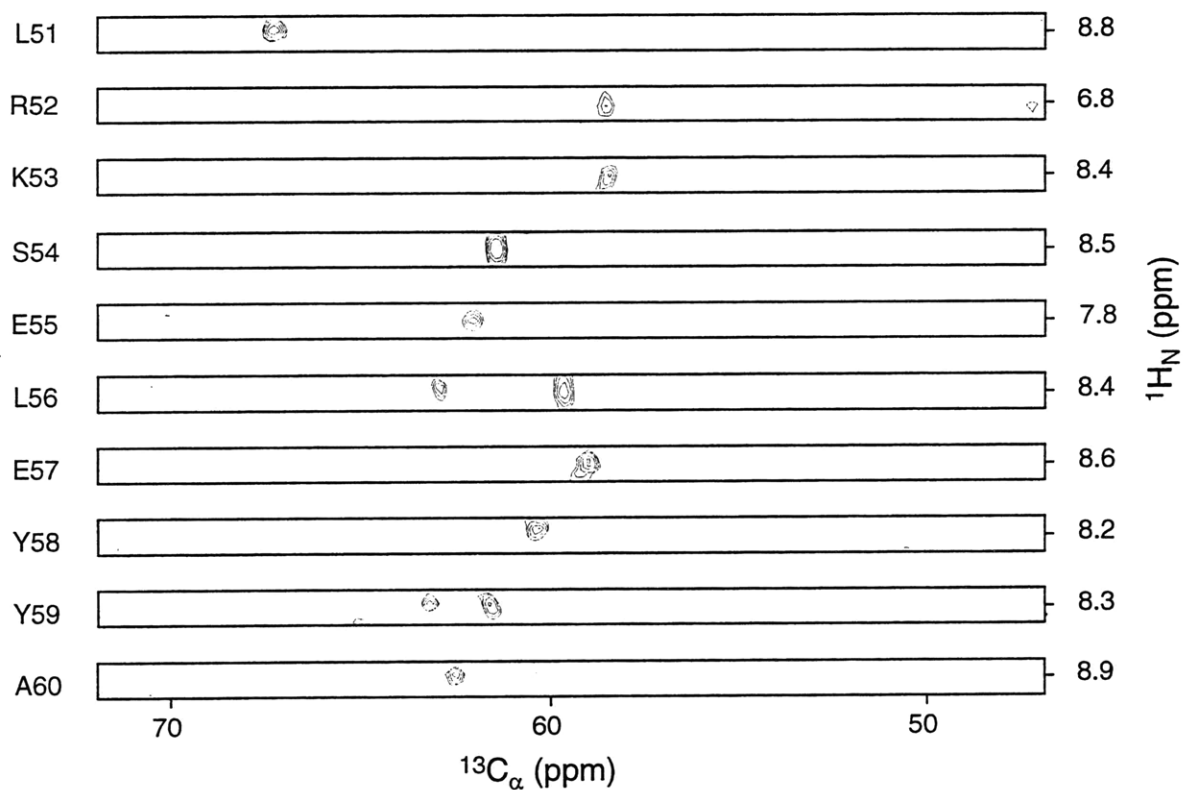
Figure 4.2.4: Selective slices of residues L51 to A60 in the 3D HN(CO)CA spectrum of $^{13}C/^{15}N$-labeled, bound form L30 protein. In each slice, the only cross peak for each amide resonance corresponds to an interresidue $H_{N(i)}$-$C_{\alpha(i-1)}$ correlation, thus allowing unambiguous identification of the C_{α} resonances.

details were similar to the HNCA experiment, except that the carbon carrier for the $^{13}C_{\alpha/\beta}$ pulses was centered at 43.6 ppm. The failure of this experiment may be due to the relatively large size of the complex that leads to large linewidths. The magnetization is on ^{13}C for a longer time as compared to the HNCA experiment (for the $^{13}C_{\alpha}$ to $^{13}C_{\beta}$ transfer), and as the carbons are known to relax faster, one would expect less signals for the large complex. Since the S/N ratio of an HNCA experiment tested on the Varian 600 MHz spectrometer was poor for the complex, the failure of the HNCACB experiment was not a surprise. In the HNCACB experiment, only a handful of residues at the N- and C-terminus gave correlations in the spectrum, while a majority of the residues showed no correlation peaks.

Besides the sequential assignments of the backbone ^{1}H, ^{15}N and $^{13}C_{\alpha}$ chemical shifts, the carbonyl ($^{13}C'$) chemical shifts were also obtained through an HNCO experiment (Muhandiram & Kay, 1994; Kay et al., 1992). This sequence correlates the amide ^{1}H and ^{15}N chemical shifts of one amino acid with the ^{13}CO chemical shift of the preceding residue, by using the one-bond $J_{N-C'}$ coupling ($\sim$ 15 Hz). A WALTZ-16 pulse train was used for both ^{1}H and ^{15}N decoupling. The C' pulses were applied at a narrow field strength of 4 kHz to ensure minimal excitation of the C_{α} resonances. The $^{13}C_{\alpha}$ 180° decoupling pulse was applied with a field strength of 9 kHz, which minimizes the perturbation of the $^{13}C'$. Since the HNCO sequence uses relatively large (about 15 Hz) coupling for correlation, the experiment acquired on the Varian 600 MHz spectrometer was very sensitive. Figure 4.2.5 shows representative slices of residues L51 to A60 from the HNCO spectrum, in which each strong cross peak represents the correlation between a backbone amide and the $^{13}C'$ (carbonyl) resonance of the preceding residue.

After obtaining the backbone $^{1}H_{N}$, ^{15}N, $^{13}C_{\alpha}$ and $^{13}C'$ assignments, attempts next was made to assign the $H_{\alpha}s$. This information was obtained through a ^{15}N-edited TOCSY-HSQC, as mentioned previously in the NOE-based assignment, and confirmed using the HNHA experiment that will be discussed later in this chapter (section 4.2.5.1).

The backbone $^{1}H_{N}$, ^{15}N, $^{13}C_{\alpha}$ and $^{13}C'$ information obtained using the triple resonance experiments not only provided starting points for the complex assignments, it was also valuable
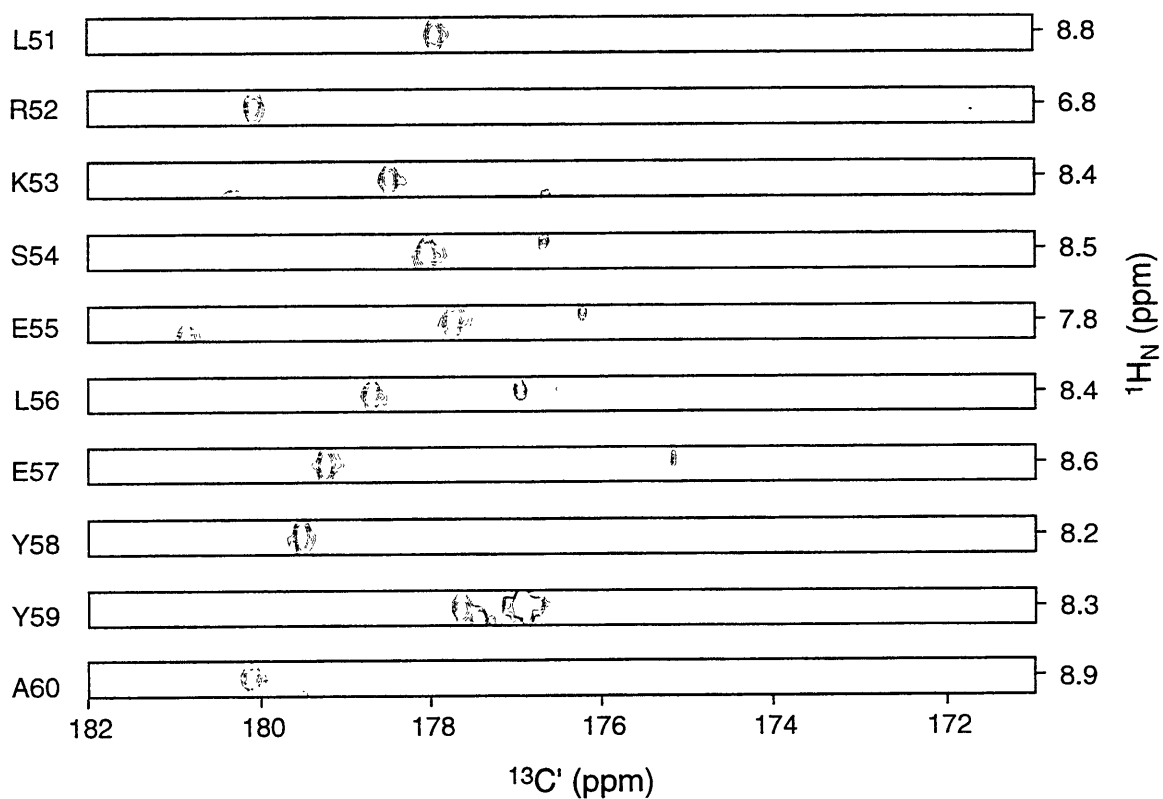
Figure 4.2.5: Selective slices of residues L51 to A60 in the 3D HNCO spectrum of $^{13}C/^{15}N$-labeled, bound form L30 protein. In each slice, the only cross peak for each amide resonance corresponds to an interresidue $HN_{(i)}\text{-}C'_{(i-1)}$ correlation.

for mapping out secondary structure using chemical shift index (Wishart & Sykes, 1994). Theoretical calculations (de Dios *et al.*, 1993) and empirical analyses of chemical shift database (Wishart & Sykes, 1994) showed that the backbone chemical shifts of ^{1}H, ^{15}N, ^{13}C$_\alpha$, and ^{13}C' are highly dependent upon the backbone torsion angles. These chemical shift values show a typical trend in comparison to the respective values in random coils for different secondary structures. Typically, ^{13}C$_\alpha$, ^{13}C' resonances of residues in α-helices are shifted downfield by an average of 2.6 ppm and 1.7 ppm, respectively, whereas in β-sheet conformations they are shifted upfield by an average of 1.4 ppm. The ^{1}H$_N$, ^{1}H$_\alpha$ and ^{15}N, on the other hand, shift upfield by an average of 0.19, 0.38 and 1.7 ppm respectively in α-helices, and shift downfield by 0.29, 0.38 and 1.2 ppm respectively in β-sheets. Thus the deviations of these chemical shifts are mapped, and a quick approximation of protein secondary structure content can be obtained.

Figure 4.2.6 shows the summary of the deviations in the chemical shifts of the protein residues in the complex form. In addition to the ^{1}H$_N$, ^{15}N, ^{13}C$_\alpha$ and ^{1}H$_\alpha$ shifts that were available for the protein in the free form, the ^{13}C' chemical shifts were obtained for the protein in the complex. As can be seen in Figure 4.2.6, the chemical shift data clearly suggest the existence of four α-helices and four β-sheets. More specifically, the secondary ^{13}C$_\alpha$, and ^{13}C' chemical shifts show contiguous segments with large downfield secondary shifts in all four helices and upfield shifts for the four strands. The secondary components mapped out with chemical shift index were found consistent with those characterized by the sequential short range NOEs in the ^{15}N-NOESY-HSQC spectrum. Among the five values, the ^{13}C$_\alpha$ secondary shifts provide detailed insight into local variation of the structure within helices. The secondary shifts are largest in the central regions of the helix, suggesting decreased stability and dynamic fraying at the ends. The ^{13}C$_\alpha$ secondary shifts of residues in helix 4 are on average smaller, showing that the helix is not completely folded in this segment and there may be residual fluctuations of the peptide chain allowing sampling of non-helical conformations.
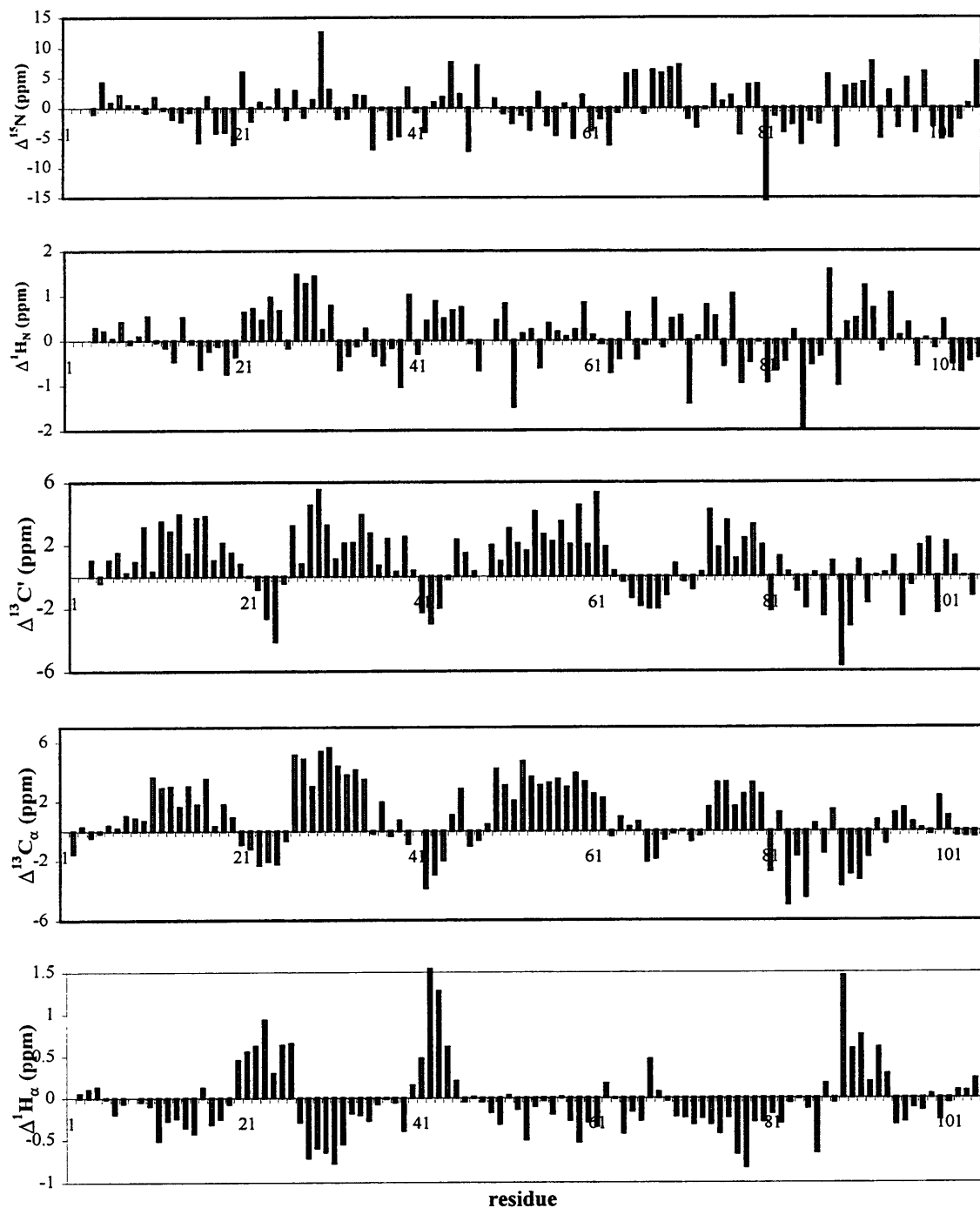
Figure 4.2.6: Summary of secondary ^{15}N, $^{1}H_N$, $^{13}C'$, $^{13}C_\alpha$, and $^{1}H_\alpha$ chemical shifts for the L30 protein in bound form. The deviation of the chemical shifts are derived from comparisons to the chemical shift index (CSI) values reported by Wishart, *et. al.*, 1994. Residues with chemical shift deviation absolute values greater than 0.1, 0.5, or 0.7 ppm for respective H_α, C', or C_α are particularly useful for identification of protein secondary structure.

4.2.3. Assignments of the side chain resonances

While the backbone sequential correlations provide some insight into the secondary structure of the protein, complete assignments of the side chains are necessary to identify all the long-range NOE correlations, which are critical for structure calculations. With the unambiguous identification of sequential backbone resonances, the side chain assignment procedure was carried out using experiments similar to those for the free form of the protein. The $^1H_\alpha$ resonance assignments were obtained in a ^{15}N-edited TOCSY-HSQC (60 ms mixing time) spectrum and were used in combination with the $^{13}C_\alpha$ assignments obtained from the HNCA and HN(CO)CA experiments to achieve through-bond side chain correlations. The side chain assignments were carried out using an HCCH-TOCSY experiment, in combination with a ^{13}C-edited NOESY-HSQC experiment acquired in D_2O described in Chapter 2, Section 2.2.2.2. The HCCH-TOCSY spectrum was acquired on a Varian Inova 600 MHz instrument with an adapted pulse sequence (Clore *et al.*, 1990) modified by Kay *et al*, 1995. This experiment produced a much better result due to the experimental settings than that for the free L30 protein acquired on the Bitter 500 MHz instrument. First, carbonyl off-resonance decoupling pulses were applied during the carbon evolution period, centered at 176 ppm using a SEDUCE-1 (McCoy & Mueller, 1992) profile with a peak amplitude of 4.72 kHz. This resulted in sharper C_α linewidths, and thus better relays were observed. Second, a shorter DIPSI-3 (Shaka *et al.*, 1988) mixing time (18 ms instead of 28 ms) was used, which reduced the net loss of magnetization due to ^{13}C relaxation. In an HCCH-TOCSY experiment, the mixing time can be optimized to compensate mainly for two factors. First, it is necessary to increase the total mixing time to see relays for the long side chains. But on the other hand, it is also critical to keep the mixing time as short as possible to avoid the rapid magnetization relaxation. There is a 2-fold reduction in signal if one of the carbons has two magnetically nonequivalent protons attached; the reduction is 4-fold if both carbons have nonequivalent protons attached (Clore *et al.*, 1990). By choosing 18 ms for the mixing period in the HCCH-TOCSY, the overall signals were

relatively strong, though complete transfers for the long side chains were not seen (*e.g.*, Lys, Arg, etc.).

In addition to a ^{13}C-edited NOESY-HSQC experiment, a CN-NOESY-HSQC (Pascal *et al.*, 1994) spectrum with simultaneous acquisitions of ^{15}N and ^{13}C-edited NOESY was recorded on the ^{13}C/^{15}N-labeled protein in the complex. A NOESY-HSQC spectrum was acquired on the Varian 600 MHz instrument with the carbon carrier centered at 43.6 ppm and nitrogen at 118.4 ppm. Decoupling of the carbonyl was achieved using a 600 Hz SEDUCE-1 decoupling pulse train centered at 175 ppm. The spectrum takes advantage of the fact that aliphatic and amide protons resonate at different regions in the spectrum. In such a spectrum, a symmetry-related cross peak can be seen for each NOE between a carbon-bound proton and a nitrogen-bound proton. This provides a rapid and straightforward approach for unambiguous assignments of most of the NOEs between aliphatic (^{13}C-attached) and amide (^{15}N-attached) protons, which are often missed in the ^{13}C-edited NOESY experiment acquired in D_2O.

Though the CN-NOESY-HSQC experiment is highly advantageous, it does have a less sensitivity because of the differences in J_{NH} and J_{CH} (J_{NH} is ~92 Hz and J_{CH} varies from ~125 Hz for methyl groups to 200 Hz for aromatic residues). In the experimental setup, the intensity of ^{13}C-modulated cross peaks was maximized ($J = 120$ Hz chosen) at the expense of some sensitivity loss for the ^{15}N-edited cross peaks. The sensitivity loss has been estimated to be about 20% (Pascal *et al.*, 1994). Moreover, the resolution of the cross peak in the ^{15}N-edited spectrum also suffered, despite the fact that extensive folding of carbon resonance was employed, with a minimum spectral width of about 3.65 kHz to allow unambiguous interpretation of NOEs. The cross peaks were observed on planes with an appropriate F1 chemical shift (either ^{15}N or ^{13}C) depending on whether the destination proton is an amide or aliphatic proton.

Complete backbone and approximately 80% of the side chain ^{1}H, ^{15}N and ^{13}C resonances were successfully assigned in the bound form L30 protein. Among these residues, all 8 Ala, 2 Asp, 8 Gly, 7 Ile, 1 Met, 4 Asn, 2 Pro, 4 Glu, 10 Ser, 7 Thr, 9 Val and 6 Tyr side chains were completely identified. In addition, 12 out of 13 Leu, 4 out of 5 Glu and 2 out of 3 Arg, were

completely identified. Only partial assignments were obtained for the remaining Leu, Glu and Arg and all the 12 Lys. The assignments obtained are listed in Table 4.1.

4.2.4. Amide exchange measurements

Upon completion of the assignment process, it is possible to analyze the secondary and tertiary structure present in the L30 protein by the presence of NOEs in NOESY spectra. In the mean time, the identification of slowly exchanging protons can be used to help locate hydrogen-bonded groups within the protein. The presence of slowly exchanging amide H_N resonances can be rationalized in terms of regular secondary structure elements (Wüthrich, 1986). Using uniformly ^{15}N labeled protein in an HSQC experiment, in which a majority of amide resonances are resolved, it is possible to simultaneously measure the exchange rates of all the slowly exchanging amide protons. The amide exchange measurements were started by dissolving the lyophilized sample of ^{15}N labeled L30 protein in complex with non-labeled L30N RNA in 99.8% D_2O at room temperature. The sample was then immediately inserted into the magnet at 30°C and allowed to come to thermal equilibrium, as judged by the stability of the deuterium lock signal. A series of ^{15}N-HSQC experiments were acquired in order to follow the intensity of cross peaks at amide positions as a function of time. A total of 16 spectra were recorded in D_2O at 30°C over a period of 20 hrs to follow the exchange. The protonation rates of individual backbone amide protons were fitted into single exponential curves to derive the exchange rates. Figure 4.2.7 shows a series of these spectra recorded as a function of time after the addition of D_2O to the protein. The first spectrum was recorded within 1 hr, starting 10 min after the addition of D_2O to the protein-RNA complex. The others were recorded after 3 hrs, 10 hrs and 20 hrs after the D_2O exchange. The amide protons that were not detectable in the first spectrum were considered to be not hydrogen-bonded. Amide protons with half-lives much longer than 1 hr can reasonably be assumed to be involved in hydrogen bonding in the interior of the protein.

Slowly exchanged amide protons within a protein can be explained by the presence of regular secondary elements (*i.e.*, α-helix and β-sheet). The amide protons and carbonyl oxygens

Table 4.1: ^{1}H, ^{15}N and ^{13}C chemical shift list of L30 protein in bound form at 30°C. Proton chemical shifts are referenced to internal TSP. Nitrogen and carbon chemical shifts are referenced to external NH$_4$Cl and TSP, respectively. The errors in chemical shifts are ± 0.02, 0.2, 0.3 ppm for proton, nitrogen and carbon, respectively. Tentative assignments are indicated in ().

Residue	N$_H$/N	C'	Hα/Cα	Hβ/Cβ	Hγ/Cγ	Hδ/Cδ	Hε/Cε	NH2/εN
A2			4.40/51.1	1.53/18.2				
P3		177.0	4.54/63.2	1.87,2.32/32.3	2.05/27.7	3.71,3.61/50.5		
V4	8.37/121.3	176.7	4.08/62.6	2.06/33.0	0.98,0.96/21.1,21.6			
K5	8.5/125.8	177.5	4.35/56.6	1.84,1.71/33.2	1.45/25.9		2.95/42.4	
S6	8.34/117.3	175.2	4.41/58.7	3.91,3.85/64.1				
Q7	8.6/122.8	176.5	4.31/56.4	2.10,1.95/29.3	2.33/34.3			7.47,6.59/111.9
E8	8.34/121.7	177.0	4.29/57.7	2.00,1.95/36.5	2.25/36.4			
S9	8.39/116.9	176.8	4.46/59.2	4.10,3.97/64.5				
I10	8.53/122.2	177.2	3.86/63.3	1.98/37.9	0.98,1.28,1.38/17.6,29.1	0.59/13.6		
N11	8.3/120.9	178.6	4.24/57.2	2.85/37.2				7.46,6.82/109.1
Q12	8.04/120.4	179.1	4.10/59.1	2.16,2.24/30.5	1.94,2.44/34.3			7.45,6.82/111.4
K13	7.83/119.7	180.4	4.12/59.7	1.54,1.64/26.3	1.82,1.92/23.3		3.06/	
L14	8.51/120.0	178.5	3.82/57.3	1.63,1.31/42.0	1.42/27.4	0.43,0.51/23.9,25.0		
A15	8.04/121.5	180.8	3.93/55.5	1.48/17.9				
L16	7.36/116.4	180.9	4.29/57.5	1.55,1.79/42.8	1.64/28.1	0.98,0.88/25.0,25.0		
V17	7.85/124.2	178.1	3.64/66.5	2.26/31.6	1.20,0.95/23.6,22.1			
I18	7.87/118.9	179.0	3.70/62.9	1.91/36.4	0.67,0.93,1.21/17.3,27.4	0.02/9.9		
K19	7.55/117.5	178.0	4.29/58.5	1.92/34.3	1.52/25.6			
S20	7.94/110.4	174.5	4.95/59.2	4.12,3.83/67.7				
G21	8.99/115.2	173.6	4.52,3.74/44.1					
K22	9/119.4	175.7	4.98/55.5	1.72,1.66/36.8	1.49,1.37/25.6		2.96/	
Y23	8.55/123.0	173.1	5.53/56.3	2.78/		6.55/133.4	6.87/118.7	
T24	9.13/113.3	171.1	4.64/61.1	3.92/70.6	1.06/19.2			
L25	8.65/125.3	176.7	4.80/53.5	1.07,1.89s/43.6	1.91/27.1	0.78,0.83/26.8,25.7		
G26	8.2/107.2	176.8	4.62,4.40/44.4					
Y27	9.57/124.9	176.5	4.00/63.7	3.04,2.82/35.9		7.06/133.2	6.93/119.3	
K28	9.55/119.9	181.0	3.65/61.5	1.68,1.18/	2.02,1.87/	1.35,1.48/	3.14,3.00/	
S29	9.73/117.8	179.2	3.91/61.3	3.82,4.01/	/			
T30	8.41/125.8	178.4	3.71/68.5	4.17/68.9	0.93/23.0			
V31	8.86/125.3	178.2	3.18/68.6	1.74/31.3	0.38,0.45/21.2,23.9			
K32	7.64/119.6	178.6	3.81/61.1	1.83,1.76/32.6	1.52,1.71/26.7		2.75,2.87/	

S33	7.96/114.7	175.8	4.12/62.1	4.01,3.64/63.6	/		
L34	7.87/124.3	181.0	3.97/59.8	1.70/42.5	1.66/27.9	0.85,0.75/27.8,24.1	
R35	8.58/121.4	179.2	4.11/59.8	1.85s,1.30/	1.43,1.55/	2.83,2.90/43.9	6.83/83.9
Q36	7.85/113.9	177.0	4.30/56.0	2.23,1.83/29.5	2.59,2.40/34.3		7.28,6.83/110.4
G7	7.81/108.9	176.0	3.96,4.07/46.9				
K38	8.13/116.3	176.8	4.31/56.4	1.91,1.52/35.6	1.64,1.32/		
S39	7.27/111.8	176.2	4.11/59.0	3.42,3.54/63.4			
K40	9.3/124.8	176.9	4.51/55.9	1.28,1.42/33.4			
L41	7.69/121.5	174.8	4.64/55.7	1.11,2.10/45.2	1.21/27.4	0.56,0.75/27.9,24.5	
I42	8.43/118.9	173.9	5.55/58.8	1.52/41.0	0.82,0.91,1.20/19.4	0.70/15.9	
I43	8.86/123.9	174.9	5.22/59.7	1.57/41.4	0.70,1.38,0.91/19.4	0.58/15.3	
I44	8.48/124.8	176.7	4.56/60.6	1.54/41.2	1.14,1.50/17.5	0.77/14.8	
A45	8.77/129.8	179.4	4.45/53.6	1.69/19.1			
A46	8.85/124.4	178.6	4.31/55.3	1.38/18.5			
N47	8.27/111.9	175.4	4.76/52.6	3.21,2.92/36.8			7.76,6.86/111.4
T48	7.5/120.2		4.31/62.5	3.93/70.5	1.50/		
P49		178.0	4.27/63.3	1.60,1.75/	2.32/	3.46,2.88/50.9	
V50	8.53/123.8	178.0	3.64/67.1	2.12/32.0	1.01,1.09/21.0,22.0		
L51	8.81/121.3	180.1	4.20/58.8	1.66,1.52/42.0	1.64/27.7	0.94,0.88/24.9,24.7	
R52	6.84/116.9	178.6	4.25/58.3	1.60,1.54/33.0	1.36,1.19/	2.30,2.60/42.8	7.14/83.8
K53	8.44/120.3	178.1	3.86/61.4	2.02,1.80/33.4	1.74,1.46/31.3	1.74/	3.13,2.91/
S54	8.54/112.8	177.8	4.40/61.9	4.01/63.1			
E55	7.8/123.8	178.7	4.26/59.8	2.50/29.8	2.49,2.62/35.7		
L56	8.37/119.3	179.3	3.96/58.9	1.16,1.42/40.2	1.62/28.0	0.71,0.96/23.9,26.7	
E57	8.6/116.7	179.6	3.80/60.2	2.02,2.30/29.3	2.64,2.20/37.3		
Y58	8.18/122.7	177.7	4.33/61.5	3.18,3.10/38.1		6.57/133.6	6.58/118.5
Y59	8.34/117.0	180.2	4.07/62.5	2.78,3.013/39.1		7.22/133.4	7.01/118.7
A60	8.94/124.3	179.1	4.06/55.8	1.39/17.3			
M61	8.23/120.3	181.1	4.18/59.1	2.27,2.42/31.2	2.74,2.52/31.9		2.08/17.1
L62	7.92/120.4	179.0	3.96/57.9	1.49,1.68/42.5	1.39/26.9	0.75,0.64/23.5,25.5	
S63	7.58/110.3	174.0	4.49/58.0	3.97,4.12/63.7			
K64	7.88/120.7	176.2	3.94/57.6	1.69,1.50/	1.39/		
T65	8.79/118.8	173.9	4.19/63.3	3.44/70.3	1.02/21.7		
K66	7.87/127.7	174.6	4.09/57.3	1.82,1.76/34.3	1.57,1.46/		
V67	7.99/121.4	175.1	4.41/61.0	1.74/34.6	0.30,0.75/21.9,21.9		
Y68	9.03/128.4	173.7	4.67/56.7	2.73,2.79/41.1		7.02/133.2	6.89/118.7
Y69	7.95/127.8	174.5	4.57/58.0	2.52,2.93/37.3		7.01/133.2	6.64/118.5

F70	8.41/126.9	176.6	4.44/57.8	2.80,2.98/41.3	7.17/132.4	7.17/132.4	7.15/132.4	
Q71	8.74/127.8	176.0	4.14/56.2	1.93,1.75/27.3	2.25,2.30/34.3			7.30,6.79/112.3
G72	6.96/107.4	172.8	3.66,4.14/44.3					
G73	8.45/106.0	173.9	4.55,4.16/44.7					
N74	9.12/118.9	179.3	4.44/55.2	3.10,2.46/37.1				7.98,7.02/113.8
N75	8.86/122.9	176.9	4.33/56.9	2.70/37.3				7.73,7.01/114.2
E76	7.84/122.3	179.6	4.06/60.0	2.26,2.37/29.6	2.49,2.32/38.1			
L77	9.03/124.2	178.2	3.51/57.3	2.33,0.68/39.0	0.89/26.4	-0.28,0.42/21.3,26.3		
G78	7.41/104.8	176.0	3.15,3.80/47.5					
T79	7.69/117.0	178.5	3.96/66.3	4.07/70.4	1.17/21.9			
A80	8.08/126.1	179.1	4.07/55.0	1.37/18.3				
V81	7.14/106.8	174.9	4.12/60.3	2.16/30.2	0.47,0.70/22.4,19.6			
G82	7.69/107.8	174.9	3.67,3.78/46.2					
K83	7.83/117.4	176.8	4.31/51.7	0.65,-0.18/33.0	1.35,1.58/			
L84	8.21/119.5	176.2	4.16/54.0	1.80,1.31/40.5	1.53/27.7	0.82,0.94/24.1,24.4		
F85	5.98/114.3	173.8	4.54/53.4	2.19,2.67/40.4	6.30/128.2	6.25/130.1	6.55/133.0	
R86	7.78/117.3	176.7	3.73/56.8	1.50,1.22/	1.50,1.03/	2.85,3.01/43.5		7.10/83.9
V87	7.64/119.7	174.6	3.89/61.5	1.29/36.1	0.41,0.54/22.6,23.9			
G88	9.93/114.7	174.6	3.92,4.04/46.4					
V89	7.08/115.8	171.4	5.40/59.3	2.22/36.1	0.89,0.96/18.8,23.3			
V90	8.47/125.8	174.0	4.53/60.1	1.64/36.1	0.64,0.70/22.5,20.9			
S91	8.79/120.3	174.7	5.25/55.0	3.89,3.92/65.3				
I92	9.2/127.1	175.2	4.14/60.9	2.11/37.1	0.74,0.95,1.27/17.5	0.65/12.3		
L93	8.7/129.9	177.1	4.77/56.4	1.32,1.71/42.5	1.52/29.2	0.74,0.66/25.9,24.4		
E94	8.18/116.3	176.3	4.57/55.9	2.05,2.26/31.3	2.30/36.0			
A95	9.16/125.0	178.4	4.04/53.7	1.42/20.1				
G96	8.46/105.9	171.1	3.69,4.14/46.5					
D97	8.76/124.9	176.7	4.65/54.7	2.88,2.43/41.2				
S98	7.73/112.4	175.6	4.36/58.4	3.80,3.92/65.6				
D99	8.42/126.0	179.6	4.80/53.9	3.01,2.61/40.1				
I100	7.83/119.9	174.6	3.69/64.9	1.76/40.6	0.69,1.23,0.98/16.2	0.51/14.3		
L101	8.44/117.0	179.3	4.12/56.7	1.59,1.78/42.2	1.53/27.4	0.75,0.92/23.1,25.6		
T102	7.64/108.3	176.4	4.44/62.8	4.37/69.5	1.18/22.0			
T103	7.47/111.3	175.2	4.43/62.7	4.19/70.2	1.15/21.9			
L104	7.53/122.8	175.9	4.40/55.3	1.43,1.52/42.4	1.50/27.0	0.50,0.50/25.6,23.9		
A105	7.72/129.8		4.18/53.9	1.40/20.8				
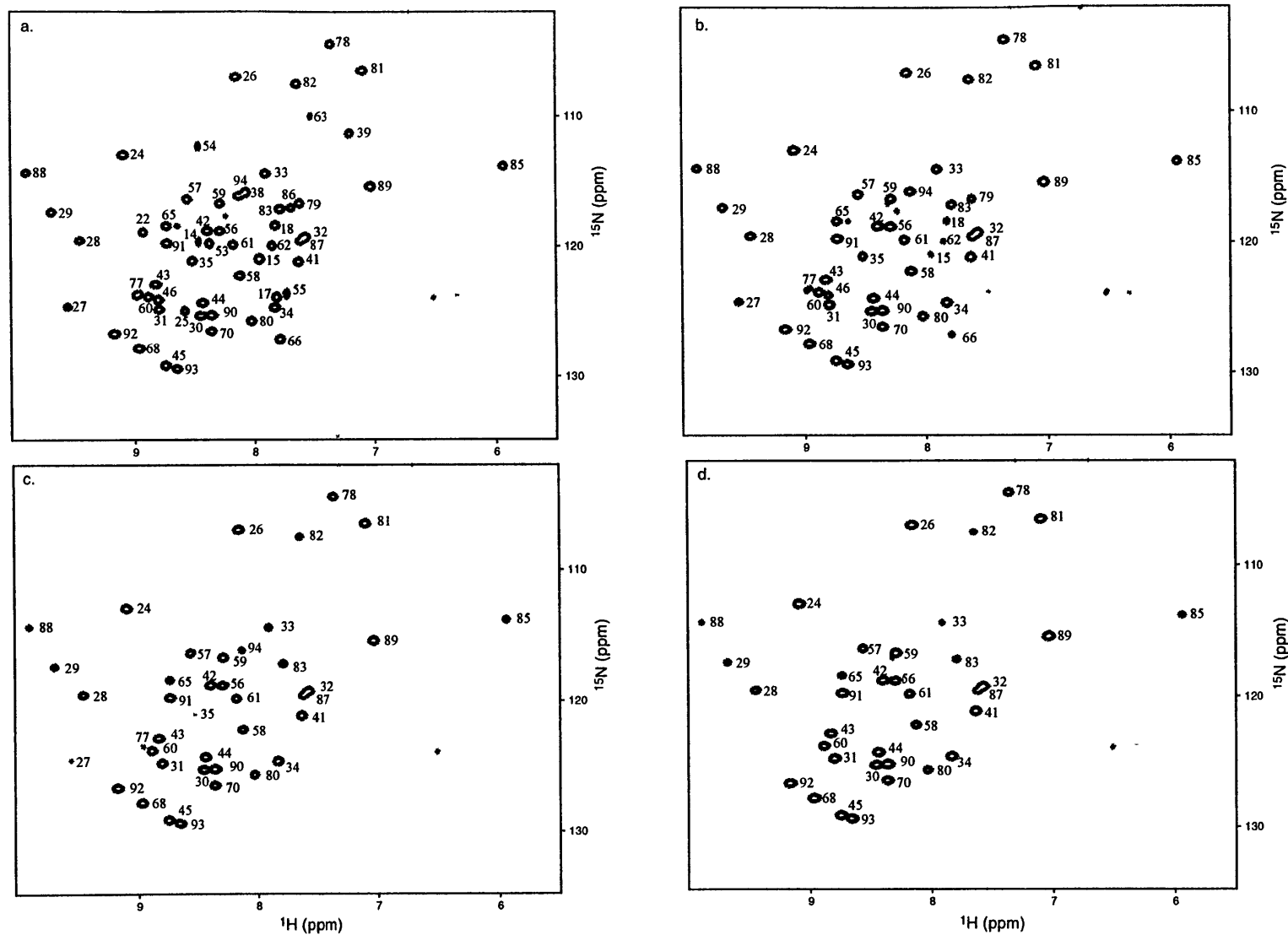
Figure 4.2.7: 1H-15N HSQC spectra of L30 protein recorded at **a**, 1 hr, **b**, 3 hrs, **c**, 10 hrs, and **d**, 16 hrs after the complex was dissolved in D2O. An observed cross peak in spectra recorded after 3 hrs of exchange suggests the possible existence of a hydrogen bond.

are involved in hydrogen bonds for all residues in a regular secondary structure, with the exception of the first four residues in an α-helix and every second residue in the peripheral strands of β-sheets. Although slowly exchanging amides can result from hydrogen bond formation, an alternative explanation could be that the proton is buried in a solvent-inaccessible region of the protein. Consequently, specific hydrogen bonds were only considered in the presence of a supporting NOE pattern. Thus, in the case of a β-sheet, a hydrogen bond can be assigned between the H_N of residue i on one β-strand to the carbonyl group of residue j on the opposite β-strand in the presence of the following NOEs: $H_N(i)$-$H_\alpha(j)$, $H_N(i)$-$H_N(j\text{-}1)$ and by $H_\alpha(i\text{-}1)$-$H_\alpha(j\text{+}1)$. Similarly, in the case of an α-helix, a hydrogen bond can be assigned between residue *i* and the residue *i*-3 in the presence of the following strong NOEs: an extended run of strong $H_N(i)$-$H_N(i\text{-}1)$, $H_N(i)$-$H_\alpha(i\text{-}3)$ and $H_\beta(i)$-$H_\alpha(i\text{-}3)$. Using the above criteria and judging from the backbone amide D_2O exchange rates, the existence of four β-strands and four α-helices within L30 protein could be interpreted.

4.2.5. Torsion angle measurements

Besides the distance information provided by analysis of NOEs, the torsion angle information that is obtained from spin-spin coupling constants is also an important source for understanding the secondary structure of the protein. Specifically, two types of torsion angle measurements (*i.e.*, backbone and side chain torsion angles) provide information about the local conformation adopted by a particular residue. These measurements are achieved through different types of experiment, and possibly at different stages during the structure determination based on stereospecific assignments.

4.2.5.1. Backbone torsion angle ϕ measurements

The H_N-H_α coupling constant is used to derive the protein backbone torsion angle ϕ, which can ultimately be used in structure calculations. Two approaches were used to obtain

accurate measurements of $^3J_{NH\alpha}$ coupling constants: 2D HMQC-*J* (Kay *et al.*, 1989a) and 3D HNHA-JR (Vuister & Bax, 1993; Kuboniwa *et al.*, 1994) experiments.

HMQC-*J* (Kay *et al.*, 1989a), also known as an HMQC-COSY experiment, estimates the $^3J_{HNH\alpha}$ coupling constants from the cross peak splittings detected in the ^{15}N cross sections of ^1H-^{15}N correlations. As the cross peak splitting is to be interpreted, the spectra had to be processed with very high resolution. In the HMQC-*J* experiment acquired on a ^{15}N-labeled L30 protein in the complex, a harsh Lorentzian-to-Gaussian apodization function was used in combination with zero-filling to 4K in the F1 dimension (^{15}N) to yield a final digital resolution of < 0.5 Hz/point. Apparent $^3J_{NH\alpha}$ values were measured directly from the cross peak splittings in the ^{15}N dimension. No simulations of line shapes or fitting the data with multiple-quantum linewidths were pursued.

The data obtained from the HMQC-*J* spectrum was more qualitative than quantitative, mainly because of the large ^{15}N linewidth associated with the protein-RNA complex. The $J_{HNH\alpha}$ coupling constants could be accurately determined for 5 residues at the N-terminus and 2 residues at the C-terminus, because of the long T_2 resulting in narrow ^{15}N linewidths. The information obtained for the remaining residues only gave a "yes" or "no" splitting answer. The smallest measured $J_{HNH\alpha}$ coupling constant measured from the HMQC-*J* spectrum was 7 Hz, and the largest one was 9 Hz. A majority of peaks did not exhibit measurable splitting. The cross peaks could be divided into three classes: peaks that clearly exhibited good signal to noise ratios; peaks that had poor signal-to-noise ratios and appeared to possess broad ^{15}N or ^1H linewidths; and peaks that could not be interpreted because of cross peak overlap. Cross peaks in the first category were assigned $J_{HNH\alpha}$ coupling constant values of < 5.0 Hz, provided that these residues also had characteristic α-helical NOE patterns. No values for the coupling constant were assumed for the other categories.

A more quantitative analysis was achieved through the HNHA experiment, which measured the $^3J_{NH\alpha}$ coupling constants from the diagonal to cross peak intensity ratios in a ^{15}N-edited H_N-H_α correlation experiment. The HNHA experiment was carried out at 30°C on the Bitter 500 MHz instrument equipped with a triple-resonance probe and z-gradient. The delay δ

and ζ were set to 4.5 ms and 13.05 ms, respectively. Solvent suppression was achieved by a water-flip back sequence. The spectrum was recorded with 512 (t_3) × 64 (t_2) × 64 (t_1) complex points in order to achieve good resolution in the ^{15}N dimension. After the data processing, the size of the absorptive part of the resulting 3D spectrum was 256(F3) × 256(F2) × 128(F1). Data analyses to obtain $J_{HNH\alpha}$ coupling constants were based on the ratio of the diagonal-peak (positive) to cross-peak (negative) intensity ratio using the following equation:

$$S_{cross} / S_{diag} = - \tan^2 (2\pi J_{HNH\alpha}\zeta) \tag{4.1}$$

where the S_{cross} and S_{diag} are the intensity of the cross and diagonal peak, ζ is set to 13.05 ms ($2\zeta \approx 1/(4J_{HH})$). The backbone dihedral angle ϕ was calculated using the Karplus equation (Karplus, 1963):

$$J(\phi) = A \cos^2(\phi - 60) + B \cos(\phi - 60) + C_\alpha\zeta \tag{4.2}$$

where $A = 6.51$, $B = -1.76$, $C = 1.60$.

There are certain corrections that need to be applied when measuring the J from an HNHA experiment. Because the H_N-H_α cross peaks and the diagonal peaks result from the anti-phase and in-phase terms, respectively, the faster relaxation of the anti-phase term attenuates the cross peak intensity relative to the diagonal peak, which results in an underestimate of the J coupling. However, the fractional error in the derived J coupling is independent of the size of the coupling and can be corrected using the available H_α flip-rate (Vuister & Bax, 1993). In addition to this, there is a relatively large error associated with glycine residues because the strong dipolar interaction between the geminal H_α protons gives rise to faster cross relaxation rates than for most other residues. This causes a decrease in the value of J measured for the larger of the two H_N-H_α couplings and a concomitant increase for the smaller ones.

Taking into account the above consideration in combination with the losses of magnetization in some resonances during the long dephasing and rephasing delays of the HNHA experiment and some overlaps of the "diagonal" resonance, the J values of 66 residues could be measured in L30 protein. No couplings were obtained from Ala2 at the N-terminus, the 2 prolines that carry no H_N protons, or 15 other residues that have short T_2 or weak ^1H-^{15}N correlations. For the other 20 residues, only a qualitative estimate of the size of the J-coupling could be made because of overlap of the diagonal ^1H-^{15}N correlations.

Besides measurements of $J_{HNH\alpha}$ coupling constants, the HNHA experiment also helped in identifying the intraresidue H_N-H_α correlations, which were useful to exclude ambiguities from the H_β resonances (*e.g.*, Sers and Thrs) identified in the ^{15}N-TOCSY-HSQC experiment.

4.2.5.2. Sidechain torsion angle χ_1 measurements

The side chain torsion χ_1 angles were derived from stereospecific assignment of β-methylene protons and γ-methyl groups. While the basic approach was described in detailed in Chapter 2, section 2.2.2.5, there were difficulties associated with stereospecific assignment of Ser β-methylene protons. Because of both overlapping with α-protons, and complications from RNA resonances, most Ser residues were not stereospecifically assigned.

Figure 4.2.8 summaries the sequential and short range NOEs, amide proton exchanges, and backbone coupling constants obtained. A complete analysis of the data to the determination of secondary structure elements has been performed, corroborating the existence of four α-helices and four β-sheets. The analysis of the NMR data resulted in a scheme for the β-sheet structure that is shown in Figure 4.2.9.

A summary of all the experiments performed on the protein in the bound form, all the data acquisition parameters were listed in Table 4.2.

<div style="text-align:center">

10   20   30   40
APVKSQESI NQKLALVIKS GKYTLGYKST VKSLRQGKSK

</div>

k_{ex}

$^3J_{NHH\alpha}$

$d_{NN}(i,i+1)$

$d_{\alpha N}(i,i+1)$

$d_{\beta N}(i,i+1)$

$d_{\alpha N}(i,i+2)$

$d_{\alpha N}(i,i+3)$

$d_{\alpha\beta}(i,i+3)$

$d_{\alpha N}(i,i+4)$

2nd Structure

<div style="text-align:center">

50   60   70   80
LIIIAANTPV LRKSELEYYA MLSKTKVYYF QGGNNELGTA

</div>

k_{ex}

$^3J_{NHH\alpha}$

$d_{NN}(i,i+1)$

$d_{\alpha N}(i,i+1)$

$d_{\beta N}(i,i+1)$

$d_{\alpha N}(i,i+2)$

$d_{\alpha N}(i,i+3)$

$d_{\alpha\beta}(i,i+3)$

$d_{\alpha N}(i,i+4)$

2nd Structure

<div style="text-align:center">

90   100   105
VGKLFRVGVV SILEAGDSDI LTTLA

</div>

k_{ex}

$^3J_{NHH\alpha}$

$d_{NN}(i,i+1)$

$d_{\alpha N}(i,i+1)$

$d_{\beta N}(i,i+1)$

$d_{\alpha N}(i,i+2)$

$d_{\alpha N}(i,i+3)$

$d_{\alpha\beta}(i,i+3)$

$d_{\alpha N}(i,i+4)$

2nd Structure

●	> 20 hrs protection
◐	3-16 hrs protection
○	1 hr protection
★	$J > 8$ Hz
☆	$J < 6$ Hz

Figure 4.2.8: Scheme of the NMR data supporting the identification of the secondary structure elements in the L30 protein. The hydrogen exchange rates, backbone coupling constance $^3J_{HNH\alpha}$, and sequential proton-proton NOE connectivities are used to identify the the secondary structure elements of the protein. The shaded bar indicates a NOE was observed, of which the thickness indicates the intensity.
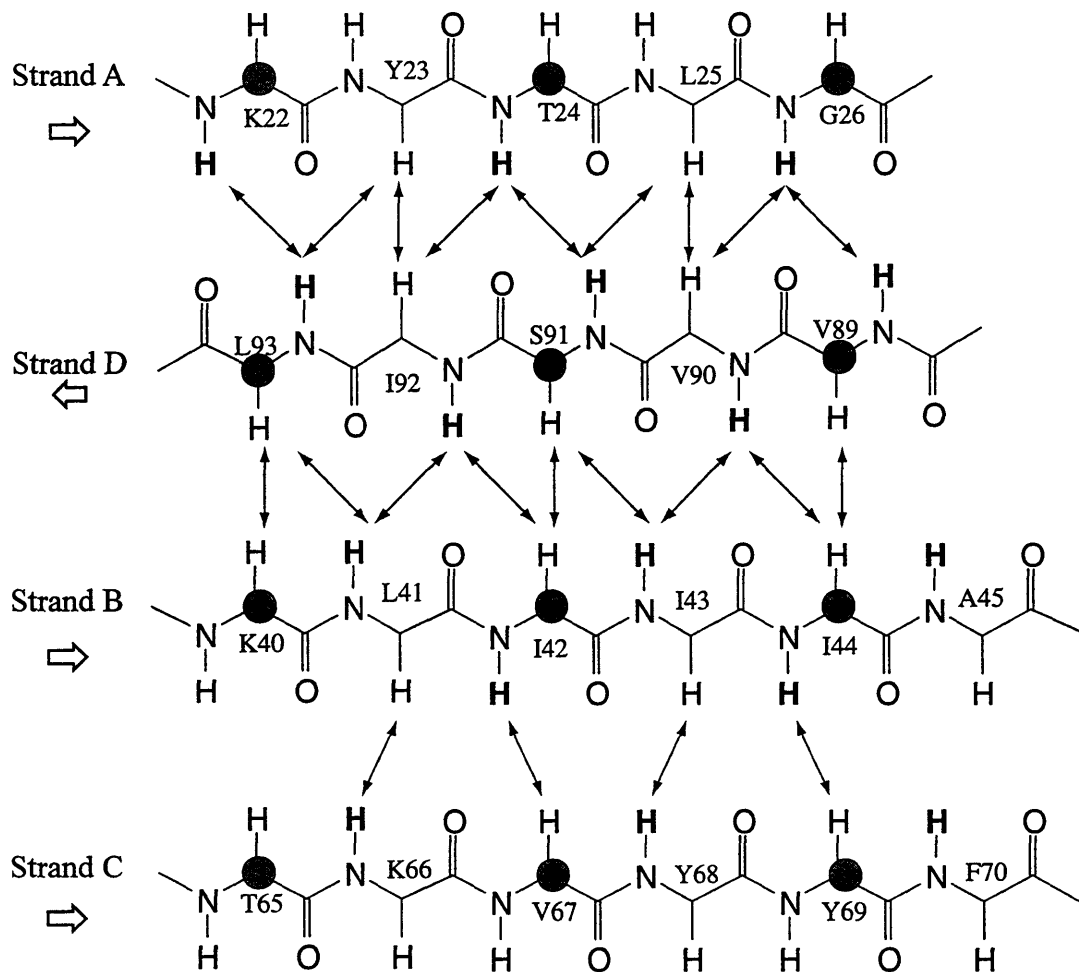
Figure 4.2.9: Schematic representation of NMR evidence for the presence of β-sheet in the L30 protein. Slowly exchanging amide protons are shown in bold. Interstrand, long-range ^{1}H-^{1}H NOEs $d_{\alpha\alpha}(i,j)$, $d_{\alpha N}(i,j)$ and $d_{NN}(i,j)$ are indicated by double-headed arrows.

Table 4.2: Data acquisition parameters and pulse sequences used in NMR experiments on L30 protein in complex. Unless specified, all experiments were acquired at 30°C. The four subdivisions in each NMR dimension (*F1*, *F2*, or *F3*) are: **a**, complex data points, **b**, spectral width (Hz), **c**, carrier position (ppm) and **d**, nuclei. In a 2D experiment, *F2* is the direct acquisition dimension, whereas *F1* is the indirect detection dimension. In a 3D experiment, *F3* is the direct acquisition dimension, whereas *F1* and F2 are the indirect detection dimensions.

Molecule	Experiment	*F3*(a, b, c, d)	*F2*(a, b, c, d)	*F1*(a, b, c, d)	T°C	Mixing time (ms)	Reference
L32 Protein /L32N RNA	NOESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H	15, 25, 30, 35	50, 100, 150, 300	
	NOESY-watergate (Bitter 501)	-	2048, 11990, 4.73, ^{1}H	512, 11990, 4.73, ^{1}H	15, 25, 30	100, 150	
	NOESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H	15, 30	100	
	dq-COSY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H			(Rance *et al.*, 1983)
	ROESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H		50	(Bax & Davis, 1985)
L32N RNA/ ^{15}N L32 Protein	FHSQC (Varain 600)	-	1024, 7000, 4.73, ^{1}H	128, 2000.0, 118.41, ^{15}N			(Mori *et al.*, 1995)
	HMQC-J (Varain 600)	-	1024, 7000, 4.73, ^{1}H	400, 2000.0, 118.41, ^{15}N			(Kay *et al.*, 1989a)
	T1ro, T1, NOE Relaxation (Varain 600)	-	512, 7000, 4.73, ^{1}H	128, 2000.0, 118.41, ^{15}N			(Dayie & Wagner, 1994)
	^{15}N Watergate NOESY-HSQC (Varian 750)	512, 9000, 4.73, ^{1}H	32, 2600, 116.34, ^{15}N	124, 9000, 4.73, ^{1}H		150	(Mori *et al.*, 1995)

	^{15}N TOCSY-HSQC-watergate (Varian 600)	512, 6999.7, 4.73, ^{1}H	128, 7000, 4.73, ^{1}H	32, 2000, 118.41, ^{15}N	60	(Marion et al., 1989)
	^{15}N HNHA-JR (Bitter 501)	512, 6000, 4.73, ^{1}H	64, 6000, 4.73, ^{1}H	30, 1600, 116.45, ^{15}N		(Kuboniwa et al., 1994)]
L32N RNA/^{15}N, ^{13}C L32 Protein	^{13}C FHSQC (Bitter 501)	-	1024, 6002.4, 4.73, ^{1}H	512, 12000, 34.83, ^{13}C		(Mori et al., 1995)
	^{13}C HSQC-arom (Varian 600)	-	1024, 6000, 4.73, ^{1}H	64, 4000, 125.55, ^{13}C		(Bax et al., 1990)
	^{13}C HSQC-CT (Bitter 600)	-	1024, 6002.4, 4.73, ^{1}H	206, 9000, 38.79, ^{13}C		(Santoro & King, 1992)
	^{13}C HSQC-CT (Varian 600)	-	1024, 7000, 4.73, ^{1}H	105, 3850, 43.64, ^{13}C		(Santoro & King, 1992)
	^{13}C HSQC-NOESY (Varian 600)	512, 6999.7, 4.73, ^{1}H	96, 7000, 4.73, ^{1}H	24, 3850, 43.64, ^{13}C	150	(Clore et al., 1990)
	^{15}N,^{13}C HSQC-NOESY (Varian 600)	512, 7500, 4.73, ^{1}H	128, 7500, 4.73, ^{1}H	28, 3850, 43.64, ^{13}C (^{15}N)	100	
	^{13}C HCCH-TOCSY (Varian 600)	512, 7000.2, 4.73, ^{1}H	128, 7000.2, 4.73, ^{1}H	32, 3850, 43.64, ^{13}C	18	(Clore et al., 1990)
	HNCA (Varian 750)	512, 9000.2, 4.73, ^{1}H	96, 5600, 61.74, ^{13}C	32, 2400, 119.85, ^{15}N		(Ikura et al., 1990)
	HNCO (Varian 600)	512, 7000.2, 4.73, ^{1}H	96, 1800, 176.18, ^{13}C	30, 2000, 118.41, ^{15}N		(Muhandiram & Kay, 1994; Kay et al., 1992)
	HN(CO)CA (Varian 600)	512, 7000.2, 4.73, ^{1}H	64, 5000, 59.0, ^{13}C	28, 2000, 118.41, ^{15}N		(Yamazaki et al., 1994)
	HNCACB (Varian 600)	512, 7000.2, 4.73, ^{1}H	64, 8000, 43.64, ^{13}C	14, 2000, 118.41, ^{15}N		(Muhandiram & Kay, 1994)

4.2.6. Backbone relaxation studies

Two-dimensional ^{15}N relaxation (T_1, T_2, NOE) measurements of L30 protein in the complex were also recorded. The pulse sequences, data size and processing parameters were similar to that described for the free form in Chapter 2, section 2.2.3. There are a few subtle differences: these experiments were recorded on a 1.1 mM protein-RNA complex sample at 30°C, with 8 T_1 relaxation delays (10.02, 20.04, 40.08, 150.3, 300.6, 601.2, 801.6, and 1202.0 ms, and repetition points of 10.02 ms and 601.2 ms), and 7 T_2 relaxation delays (2.55, 5.10, 10.21, 20.42, 38.28, 61.25, 96.98 ms, and repetition points of 2.55 and 10.21 ms). The heteronuclear NOE (^{1}H-^{15}N) relaxation were measured by two spectra, one with the NOE effect, and one without. The T_1 and T_2 relaxation rates were extracted from fitting cross peak intensities to a single exponential decay curve, and the heteronuclear NOEs were measured by the ratio of peak intensities between with-NOE/without-NOE spectra as described in Chapter 2, section 2.2.3.

Figure 4.2.10 shows the relaxation parameters T_1, T_2 and NOEs measured for the L30 protein in the bound form. Similar to the protein in the unbound form, nine residues at the N-terminus and five at the C-terminus are disordered because of relatively rapid motion, which are expected for the terminal regions. In addition, G73 also shows greater motion than the surrounding residues because of the flexible G72-G73-Asn74-Asn75 linker. Despite the flexibility at the termini and linker, the rest of the residues in the protein show similar relaxation rates, which suggest homogeneous motion. The largest difference between the free and bound form L30 protein is observed in the region between residues 74-88. This region is relatively flexible in the free form, as judged by either exchange broadened or missing correlation peaks, relaxation rates on the millisecond time scale. Upon RNA binding, not only do all of these resonances display observable correlation peaks, the relaxation rates can also be accurate determined (except those that have overlapping chemical shifts). The measurable T_1, T_2 and NOE relaxation parameters confirm the stabilization of this region upon complex formation.
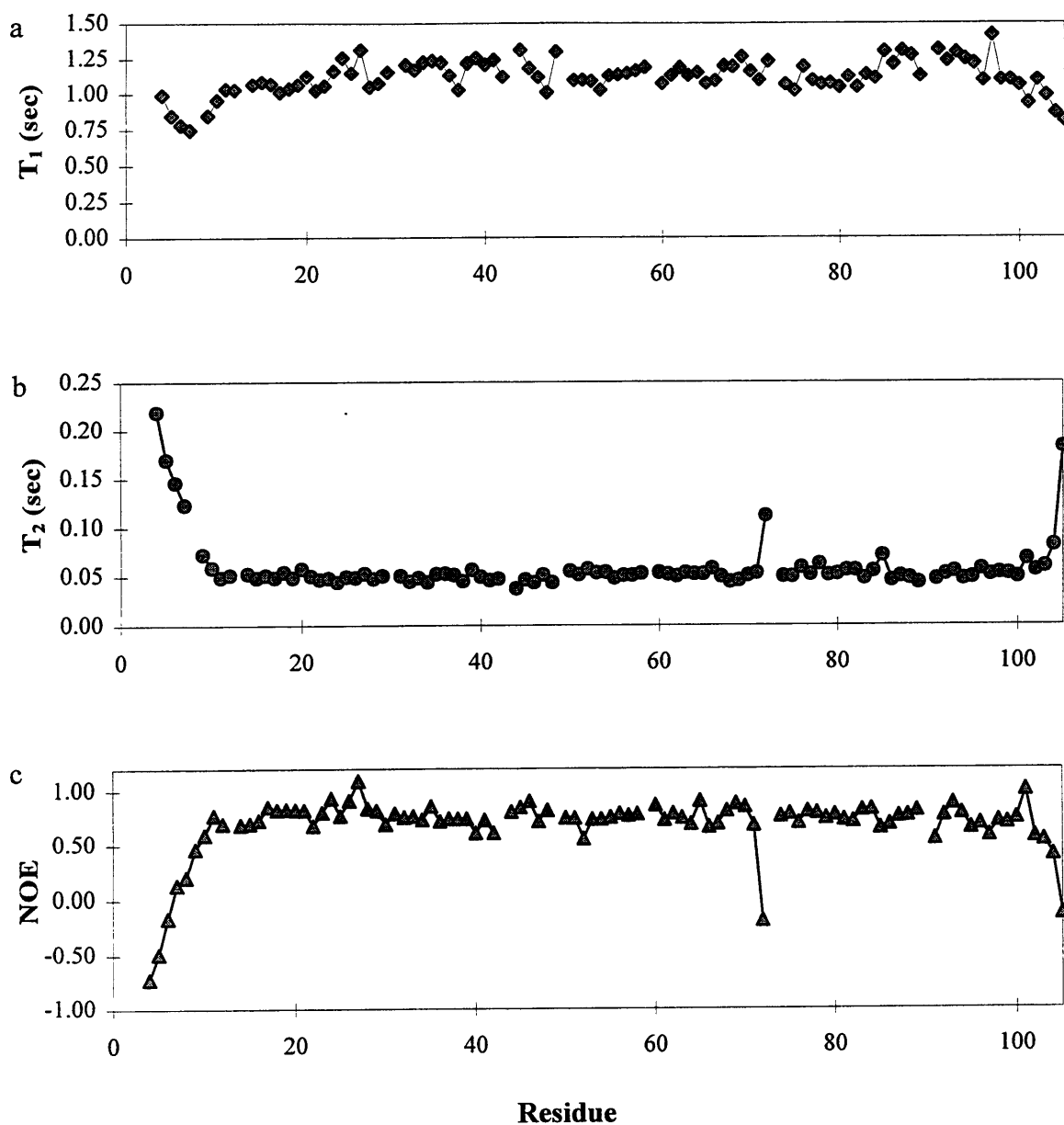
Figure 4.2.10: Plots of backbone relaxation parameters T_1, T_2, and NOE of the bound form L30 protein as a function of residue number. The data were recorded with a 600 MHz spectrometer at 30°C. Measured T_1 values are shown in panel **a**, T_2 values in panel **b**, and ^{15}N-^1H NOE data in panel **c**. The missing data points result from overlapping cross peaks in the ^{15}N-edited HSQC spectrum.

In conclusion, the formation of the L30 protein and L30N RNA complex was carried out by titration at a low concentration. A millimolar concentration complex sample was obtained by gradual concentration, which enabled the subsequent NMR analysis of the complex. The unambiguous assignments of almost all backbone resonances (^1H_N, ^{15}N, $^{13}C'$, $^1H_\alpha$ and $^{13}C_\alpha$) were achieved using a series of triple resonance experiments, and the assignments of 95% of the side chain ^1H, ^{15}N and ^{13}C resonances were obtained using heteronuclear-edited TOCSY and NOESY experiments. The backbone torsion ϕ angles for 70% of the residues have been derived from $J_{HNH\alpha}$ measurements obtained from 3D HNHA and 2D HMQC-J experiments. The analysis of sequential NOEs, chemical shift alignments of various backbone resonances, backbone D_2O exchange rates, and backbone $J_{HNH\alpha}$ coupling-constant measurements confirmed the existence of four α-helices and four β-strands within the protein in the bound form. Comparison of resonance chemical shifts and sequential NOEs of the protein suggests that the overall folds are similar in the free and bound form with a few local conformational reorganizations. However, the protein in the bound form is significantly stabilized by the presence of the RNA. As compared to the protein in the free form, the bound protein has a substantially increased thermal stability, completely observable backbone resonances, and almost homogenous relaxation rates for the four α-helices and four β-strands.

Chapter 5. The L30N RNA in the Bound Form

5.1. NMR Characterization of the Bound Form L30N RNA

The next step after the protein assignments is the correlations of the L30N RNA resonances in the bound form. Since the RNA has been characterized in the free form, similar buffer conditions (10 mM potassium phosphate, pH 6.0-6.5, 0.02% NaN$_3$ and 0.1 mM EDTA) were used for the bound form RNA. Initial temperature dependence analyses of proton 1D NMR spectra show that the RNA proton resonances have very broad linewidth at temperatures below 20°C, and the resolution increases with elevated temperatures. In order to achieve better resolution for most RNA resonances, and also to be consistent with the assignment conditions for the L30 protein in the bound form, 30°C was used for most experiments. Various 2D experiments with different temperatures were acquired to resolve resonances with degenerate chemical shifts, which also assisted the assignment process.

5.1.1. Use of mutants to assist the assignment of the RNA

The relatively large size of the L30 protein-RNA complex has been a challenging NMR project, especially for the characterization of L30N RNA in the bound form. While the traditional NOE-based assignment procedures are useful for A-form RNA, the sequential NOE connectivities occasionally break down at non-standard conformations. Recently, sequence specific assignments of an RNA can be achieved by triple resonance experiments. However, in the case of L30 RNA in the bound form, the large size and the chemical anisotropy effects associated with aromatic resonances limited the application of many triple resonance experiments. Despite the limitations, the use of isotopically labeled RNAs (^{13}C/^{15}N uniformly labeled RNA, or ^{13}C/^{15}N-guanine-only labeled RNA) greatly facilitated the assignment procedure, and mutation studies were also very useful in resolving ambiguities in the spectra.

First, it is necessary for the mutants to be equally active as wild type, so as to justify their usefulness in the assignment. Active mutants U14C, A55C, A55U and A57C RNA were selected on the basis of *in vitro* selection results (Li & White, 1997). In all these mutants, the individually changed residue did not affect the protein binding affinity dramatically. The mutant RNAs used in the NMR studies each carry a point mutation. The binding affinities of these mutants were comparable to that of the wild type (12 ± 4 nM) as determined in gel shift assays. Also, the backbone resonances, as determined in the ^{15}N-edited HSQC spectra of the L30 protein, displayed similar resonance shifts, indicating the similarities in the complex structures of all the three mutants (U14C, A55C and A55U) (Figure 5.1.1). Only minor differences (± 0.1 ppm) were observed for protein residues close to the RNA mutation (if any). For the A57C complex, since the protein was unlabeled, no HSQC spectrum was recorded. For the RNAs, the homonuclear 2D H_2O NOESY, D_2O NOESY, TOCSY and COSY spectra of all the mutants showed the preservation of the overall RNA conformation. Typically, in the aromatic-sugar region of the NOESY, no chemical shift differences greater than 0.2 ppm have been detected. A few significant chemical shift changes were observed in the RNA imino resonance region, and these can easily be attributed to the mutation itself or by ring current effects of the mutation. These results indicate that the mutations adopt similar conformations as wild type, and thus helped in assignments by the small chemical differences that was generated by the mutations.

5.1.2. Assignment of the exchangeable protons

The assignments of the exchangeable protons were made using both homonuclear and heteronuclear experiments. In addition to the standard homonuclear NOESY spectra acquired in H_2O, heteronuclear 2D correlation spectra were recorded using either uniformly ^{13}C/^{15}N-labeled, or ^{13}C/^{15}N-guanosine-only labeled L30N RNA in complex. Standard ^{15}N-edited FHSQC (Mori *et al.*, 1995) spectra were acquired at 11, 15 and 25°C. These were recorded by centering the ^{15}N transmitter either around 117 ppm to obtain the fully exchangeable ^{1}H-^{15}N correlations, or around 150 ppm to obtained the imino ^{1}H-^{15}N correlations. The evolution period was optimized for $J_{HN} =$
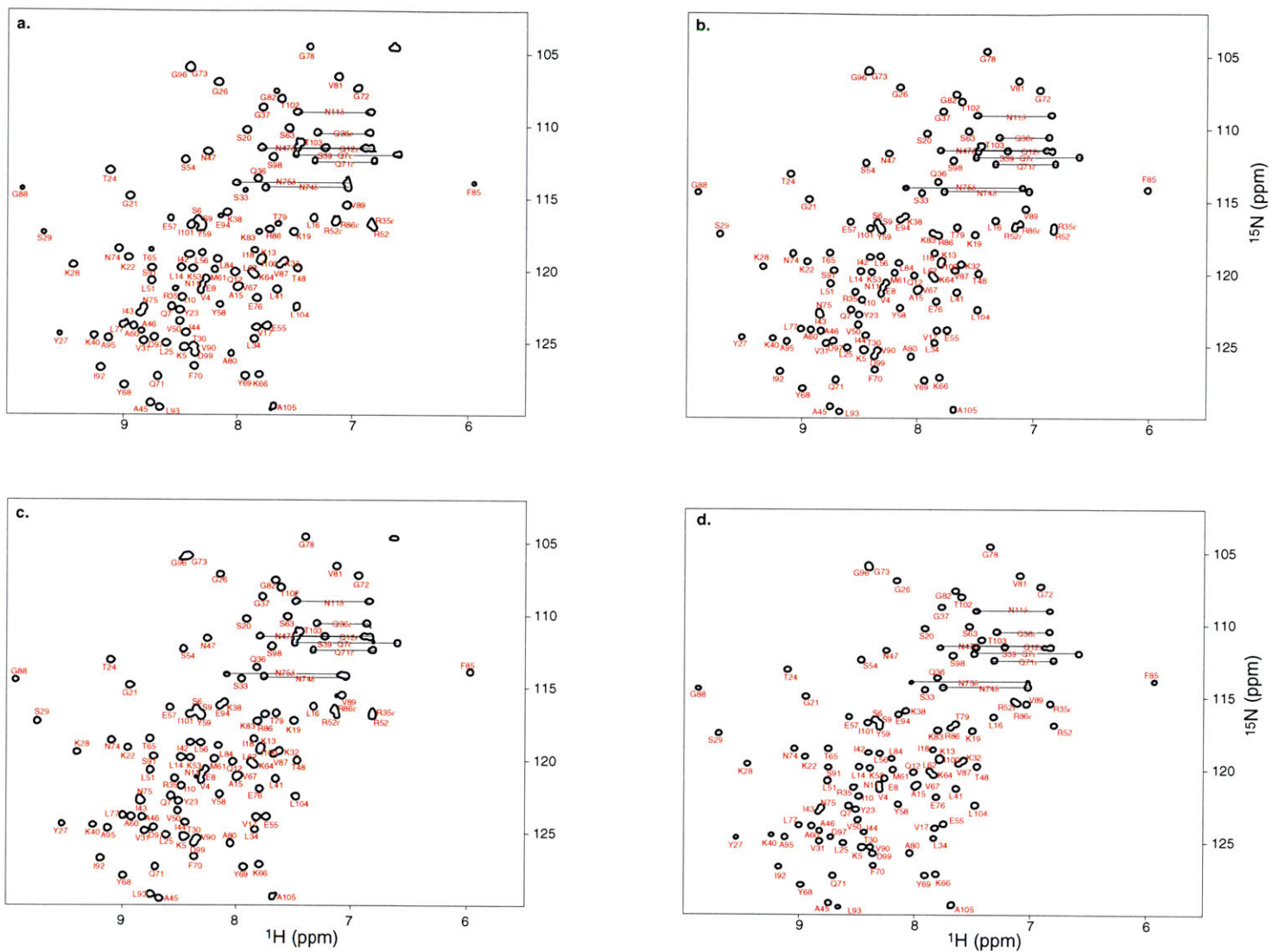
Figure 5.1.1: ^1H-^{15}N HSQC spectra of the L30 protein in complexes with **a**, L30N, **b**, A55C, **c**, A55U, and **d**, U14C RNAs. Not that the Arg ε side-chain peaks in **d** are folded differently than those in **a**, **b** and **c** because of different spectral widths in the ^{15}N dimension.

90 Hz. Furthermore, 3D CN-NOESY-HSQC spectra were recorded by centering the ^{15}N transmitter around 120 ppm and ^{13}C around 104 ppm to observe the nonexchangeable aromatic and H1' protons. A ^{15}N NOESY-HMQC spectrum was recorded at 750 MHz centering ^{15}N to 150 ppm to observe imino-proton NOEs.

5.1.2.1. Imino resonance assignments

The imino proton resonances of L30N RNA showed no significant changes in a homonuclear spectrum upon complexing with protein. The only exception was the appearance of one additional imino resonance at 11.18 ppm. Even the NOE pattern of these imino resonances did not show a large change on complex formation (Figure 5.1.1, panel b). The imino-imino NOEs between the stacked base pairs were easily observable even though the resonance linewidths were broader compared to those in the free form. These imino protons correspond to the two helical regions, suggesting the preservation of two helices upon protein binding. The imino-imino NOEs were observed between G6-U63-G62-G61 within the lower helix, and G13-U14 and G53, and G15-U16-G50 within the upper helix, similar to that observed in the free form of the protein.

Though the helices were similar in the free and bound RNA, the imino resonances displayed larger differences in the internal loop region. These differences were particularly clear in an ^{1}H-^{15}N HSQC spectrum (Figure 5.1.2, panel **a**) which shows the appearance of another additional guanine imino resonance with a further upfield shift (9.0 ppm). This resonance could not be observed in a homonuclear experiment as it was masked by the protein amide resonances. Furthermore, the imino protons of G10, G13 and G61 flanking the internal loop also displayed slight differences in their chemical shifts, indicating the conformational changes within the internal loop. No further change was observed for the imino resonance of U60 that forms a wobble base pair with G10 in the free form at low temperature (< 10°C). Unfortunately, it was not possible to go below 10°C to observe this peak, as the linewidth became too broad.
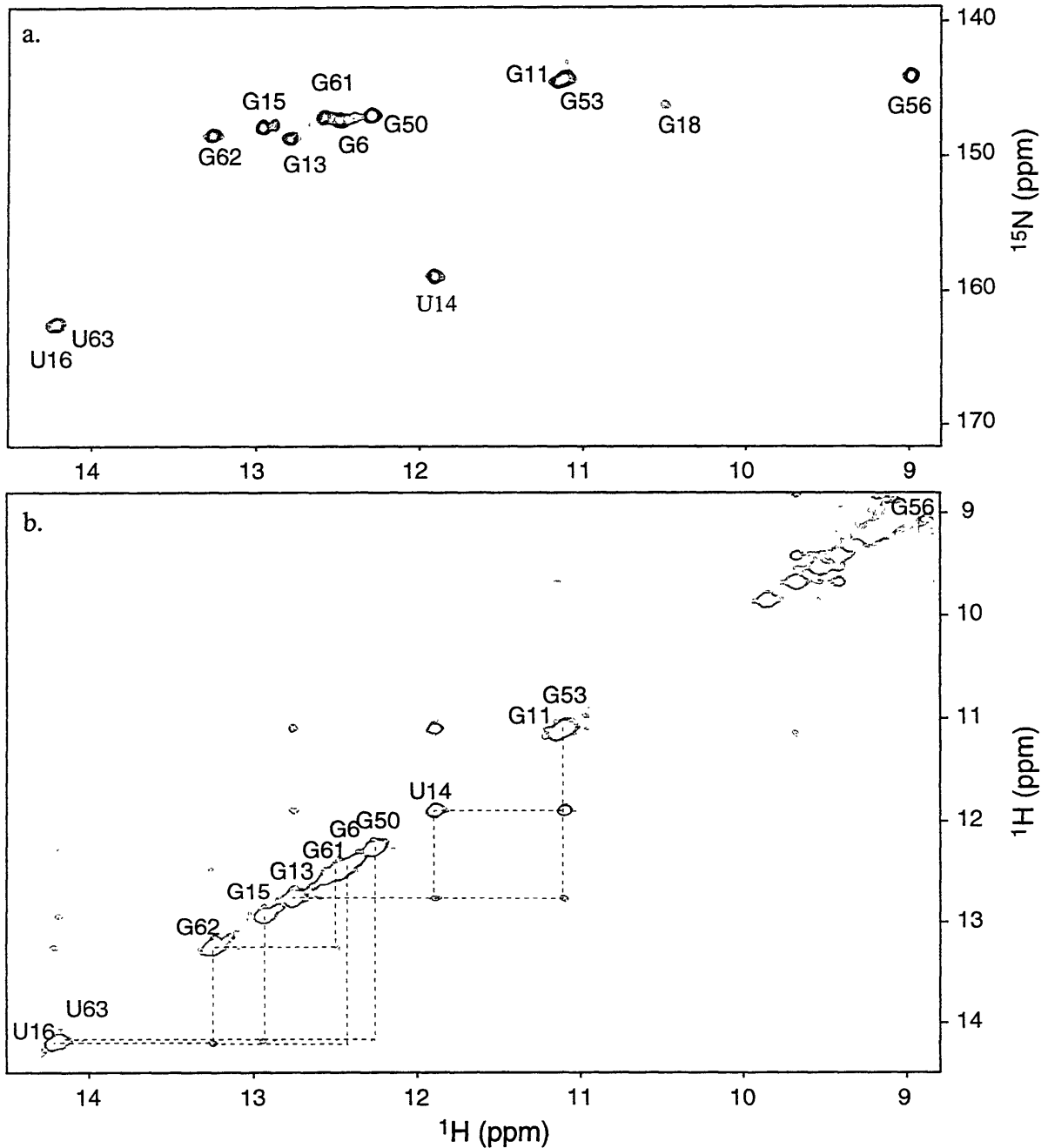
Figure 5.1.2: Imino proton spectra of the L30N RNA. **a**, A ^{1}H-^{15}N HSQC of the uniformly ^{13}C/^{15}N-labeled RNA, and **b**, a 100 ms proton NOESY of the RNA in the complex. These spectra are recorded at 30°C in H_2O containing 10 mM potassium phosphate, pH 6.5, 0.1 mM EDTA, and 0.02% sodium azide. The concentrations of labeled and unlabeled RNA were 0.8 mM and 1.6 mM, respectively. The imino peaks are labeled with the assignments in each spectrum, and dashed lines trace the sequential imino-imino NOEs in panel **b**.

The assignments of the two additional imino protons that appeared in the complex were particularly challenging, not only because there were no sequential imino-imino NOE connectivities, but also because they showed no conventional NOE patterns (*i.e.*, regular Watson-Crick or wobble base pairs) for base pair identification. Although an HCCNH-TOCSY type of experiment would have been ideal to correlate the exchangeable and nonexchangeable proton, no signals were observed for the HCCNH-TOCSY experiment. This was expected due to the short T_2 resulting from chemical shift anisotropy relaxation of the aromatic protons and intermediate exchange properties of the residues.

Thus to carry out the assignment of these imino protons, the NOE-based approach was again employed for the assignments of all the nonexchangeable protons were obtained (will be discussed below in section 5.1.3). The resonance at 11.18 ppm was assigned to G11 based on internucleotide and intermolecular NOEs, which included a strong NOE to 7.38 ppm belonging to the G56 H8. A strong NOE to the G56 H8 and no NOE to its own amino protons suggest that G11 and G56 form a reverse Hoogsteen type of base pair involving the G11 imino proton and the exocyclic amino proton forming hydrogen bonds to G56 N7 and O6, respectively. The resonance at 9.0 ppm to G56 was assigned to G56 though the NOE patterns were more complex. The peak was initially ignored in the homonuclear NOESY experiment because of the overlapping protein resonances and lack of interbase imino-imino NOEs. The NOEs of the imino resonance at 9.0 ppm were identified only in an ^{15}N-edited NOESY-HMQC experiment, and only one of these was to A12 H2. Most of the other NOEs were to the Phe85 resonances of the protein. Thus, at this stage it is reasonable to that the G56 might form a hydrogen bond with the protein.

5.1.2.2. Exocyclic amino resonance assignments

Since all cytosines are located outside of the internal loop, it was not surprising that the amino proton resonances do not undergo significant changes upon protein binding. All but the C19 and C65 amino protons were assigned according to strong NOEs to the imino protons in the Watson-Crick pairs, and cross-checked with NOEs to their H5s once the nonexchangeable proton

were assigned. The chemical shifts of the attached ^{15}Ns were assigned according to their proton

NOEs in a ^{1}H-^{15}N NOESY-HSQC spectrum.

Compared to the cytosines, amino protons of the guanines and the adenines display

additional resonances upon complex formation, which is expected as these are in the internal

loop region. Unfortunately, all the amino proton peaks are significantly broader than those in the

free form, and thus NOE-based assignments were unsuccessful. An ^{1}H-^{15}N CPMG-NOESY

(Mueller *et al.*, 1995) spectrum was acquired to assist in the correlation of these exchange

broadened amino peaks, but no improvement was observed in this spectrum.

5.1.3. Assignment of nonexchangeable resonances

In contrast to the exchangeable protons, the nonexchangeable protons display dramatic

and unusual changes for the residues within the internal loop region upon the complex formation

with the protein. The changes are particularly significant in the aromatic-anomeric region of the

homonuclear NOESY spectrum (Figure 5.1.3). In addition to the sequential NOE walks in the

helices that had previously been established in the free RNA, more sequential NOE cross peaks

which were identified to belong to the internal loop region were observed in a homonuclear

NOESY spectra. These additional resonances were more obvious in a ^{13}C-edited HSQC

spectrum, many of the resonances showed large chemical shift changes. Some of these are

unusually downfield, such as peaks at 8.8 ppm in the base region, at 6.8 ppm for H1' and at 5.22

ppm for H4'. The increased dispersion of chemical shifts and new NOEs within the internal loop

suggested formation of a more regular structure.

Two types of isotopic labeling patterns were introduced to facilitate the RNA

heteronuclear NMR experiments. These were a uniformly ^{13}C/^{15}N-labeled and ^{13}C/^{15}N-

guanosine-only labeled L30N RNA. While the ^{13}C/^{15}N-labeled sample was used to identify all

^{1}H, ^{13}C, ^{15}N assignments of RNA, the ^{13}C/^{15}N-guanosine-only labeled sample was used to assist in

the purine assignments in the internal loop.
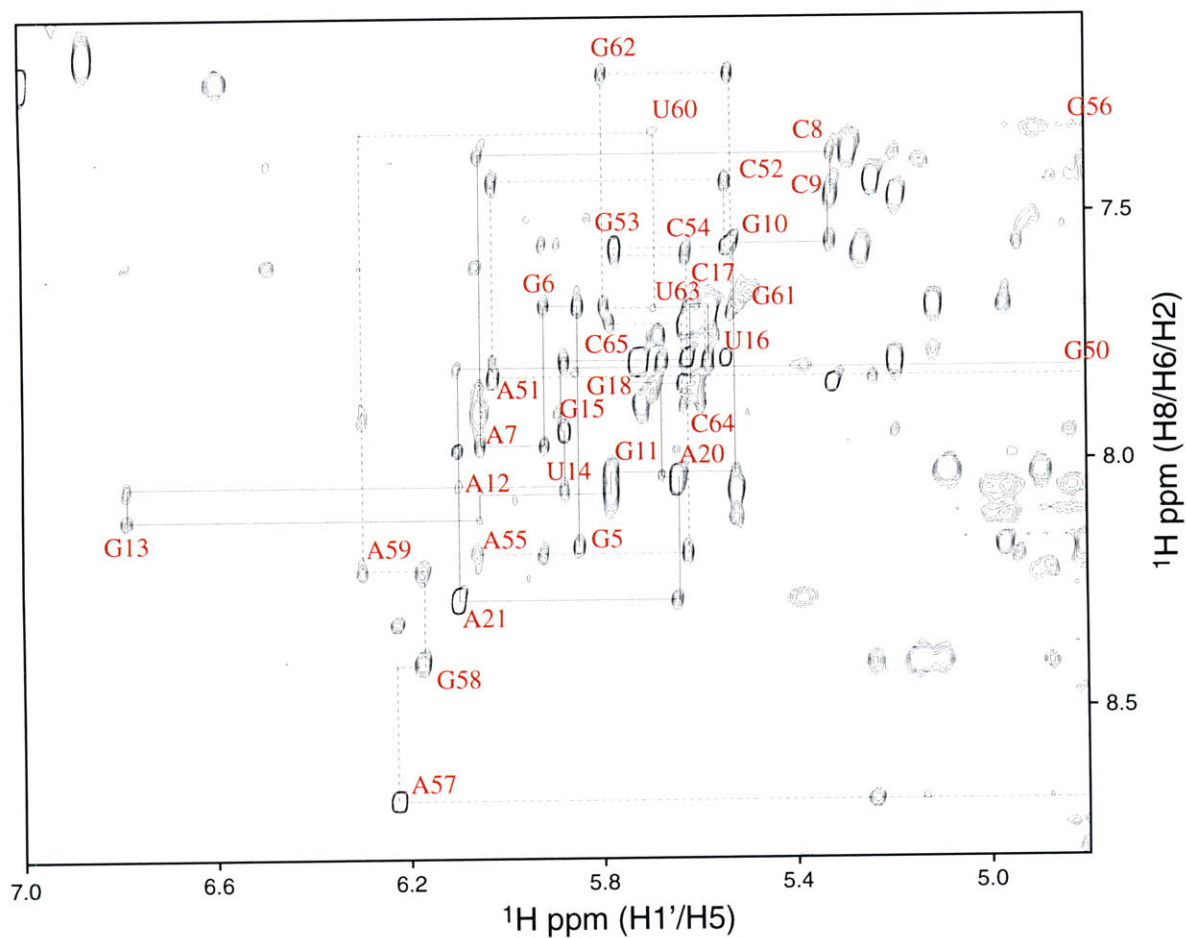
Figure 5.1.3: Aromatic (H8/H6/H2) and ribose H1' proton regions of a 300 ms NOESY spectrum of L30N RNA in the complex recorded at 30°C. The solid lines trace the sequential NOE connectivities from G5 to A21, and dashed lines trace the connectivities from G50 to A55 and A57 to C65. The labels indicate intranucleotide H8/H6 to H1' NOEs. The G51 and G56 H1' resonances are shifted downfield to 4.01 and 4.63 ppm, respectively, and are not included in this plot.

The following experiments were acquired for the nonexchangeable proton assignments. Constant time ^{13}C-HSQC spectra with the carbon offset at 83 ppm were recorded at 25 and 30°C. The evolution periods were optimized for $J_{HC} = 200$ Hz, and the constant-time period was set to $1/J_{CC}$ (about 27 ms). Because of the relatively low concentration of the ^{13}C/^{15}N-guanosine labeled RNA sample, 3D NOESY and TOCSY experiments were acquired only on the uniformly ^{13}C/^{15}N-labeled sample. A 3D CN-NOESY-HSQC spectrum was acquired with the ^{15}N transmitter around 120 ppm to observe NOEs originating from all exchangeable protons, and ^{13}C around 104 ppm to observe NOEs from nonexchangeable aromatic and H1' protons, respectively. Various 2D and 3D experiments were recorded with the sample in D$_2$O. These included 2D ^{13}C-HSQC spectra at 15, 25 and 30°C. The HSQC spectra were acquired by either centering the ^{13}C transmitter at 116 ppm for the aromatic ^{1}H-^{13}C correlations, or around 83 ppm to obtain sugar ^{1}H-^{13}C correlations. A 3D ^{13}C-HSQC-NOESY spectrum with 150 ms mixing time and a 3D HCCH-TOCSY with 12 ms mixing time were as recorded for sugar assignments, with the ^{13}C transmitter around 83 ppm for the sugar proton assignment. While the HCCH-TOCSY and CN-NOESY-HSQC spectra were acquired at 600 MHz, ^{13}C HSQC-NOESY spectrum was acquired on a 750 MHz spectrometer. Two additional experiments were also acquired to assist in the assignments. A ^{13}C HSQC-NOESY spectrum with the ^{13}C-carrier at 140 ppm, in the middle of the C8/C6 and C2 region, was acquired on a 600 MHz spectrometer to observe aromatic protein NOEs.

Although a series of triple resonance sequences designed to correlate either sugar to aromatic or aromatic exchangeable to nonexchangeable resonances have become available (Simorre *et al.*, 1995; Fiala *et al.*, 1996) in recent past, they were not successful for the L30N RNA. The main problem was due to the fast decay of the signal that was apparent with the small overall correlation time of the complex. Besides this, conformational exchange and chemical shift anisotropy effects on the aromatic nuclei further reduce T$_2$' (less than 25 ms for the internal loop region).

5.1.3.1. The nonexchangeable proton resonances in the stem

The resonance assignments of two helical stems were carried out in a similar manner as for the free form. Since many resonances show similar NOEs and chemical shifts, the assignments were straightforward and were obtained by the combination of sequential walks in homonuclear NOESY (Figure5.1.3), ^{13}C-NOESY-HMQC and HCCH-TOCSY experiments.

5.1.3.2. The nonexchangeable proton resonances in the internal loop

The peak patterns for the residues in the internal loop of the RNA were much different in the complex as compared to the free form. Many distinct peaks were observed in an HSQC spectrum, which showed a wide chemical shift dispersion (*e.g.*, ranging from 4.0 to 6.8 ppm in H1' resonances). Furthermore, many interbase NOEs were observed in the NOESY spectrum. Though it was possible to make sequential assignment on the basis of NOEs, the assignments were not foolproof since the internal loop leads to unusual NOE features that could mask the correct assignment. Thus the final assignment was made with the help of the ^{13}C/^{15}N uniformly labeled and G-specific labeled L30N RNA samples, and by using the four RNA mutants. The G-specific labeled RNA was particularly important in differentiating the purine aromatic resonances within the internal loop that is rich in purines (containing four adenosines and four guanines). Comparison of the HSQC spectra between the fully labeled RNA and G-only labeled RNA allowed unambiguous identification of all G-aromatic resonances within the internal loop (Figure 5.1.4). The RNAs with point mutations were also important for sequential assignments within the internal loop, which will be discussed along with the assignment process.

The assignments were initiated by analyzing the base to sugar proton regions. Briefly, sequential walks for the base protons (H8/H6) to ribose H1' protons of G10 to G13 and C54 to G61 within and flanking the internal loop of the complex were obtained on homonuclear NOESY spectra acquired at various mixing times. Spin system information (*e.g.*, G vs A, H1' vs H4', *etc.*) was supplemented with the corresponding 2D ^{13}C-edited HSQC experiment; and sequential connection with degenerate chemical shifts were verified using a 3D ^{13}C-edited NOESY-HMQC spectrum. Weak connectivities at the G10-G11, A12-G13, A59-U60-G61, and broken
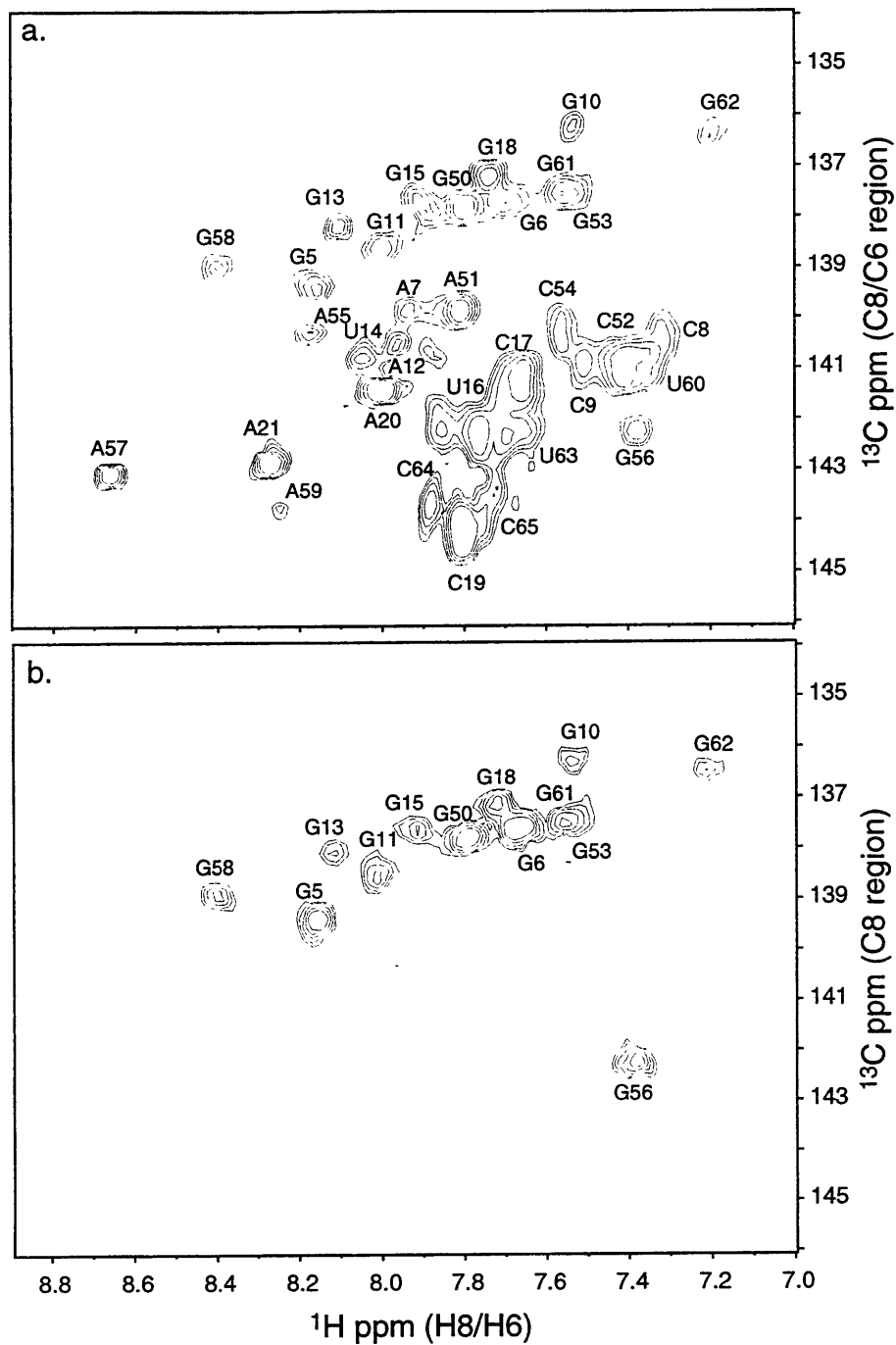
Figure 5.1.4: Aromatic (H8/H6 and C8/C6) regions of ^{1}H-^{13}C HSQC spectra acquired on **a**, uniformly ^{13}C/^{15}N-labeled L30N RNA and **b**, ^{13}C/^{15}N G-only labeled L30N RNA in the bound forms. The guanine residues can be unambiguously identified in the G-only labeled spectrum. Note that G56 has an unusual downfield ^{13}C chemical shift.

connections at A55-G56-A57 were observed within the internal loop. One of the most noticeable features was the existence of three sets of unusual medium to medium-strong sugar H1'-H1' NOEs between A12 and G13, G13 and A59, and A55 and A59 (Figure 5.1.5, panel **a**). None of these NOEs are due to spin-diffusion, as they can be seen even in NOESY spectra with a 50 ms mixing time. It was initially speculated that these NOEs might have been a result of conformational exchange, but subsequent experiment suggested otherwise. A ROESY spectrum confirmed that these peaks were true NOEs and not due to exchange, as the true NOE peaks have the opposite sign to the diagonal peaks, while exchange peaks have the same sign as the diagonal in a ROESY spectrum (Cavanagh *et al.*, 1996; Battiste, 1996). The assignment of the peak at 6.78 ppm was confirmed by the $^{13}C/^{15}N$ G-only labeled sample. In addition, the point mutation of A57 to C, and A55 to either C, or U showed preservation of the observable NOE patterns (Figure 5.1.5, panel **b**, **c** and **d**). The assignment of the G13 was based on sequential A12 H1'-G13 H8-G13 H1'-U14 H6 NOE connectivities, and on the direct chemical shift changes (from 6.78 to 6.65 ppm) that occur between L30N wild type and a U14 to C mutation (data not shown). The resonance at 6.05 ppm was assigned to A55 according to the sequential C54 H1'-A55 H8-A55 H1' NOE connectivity, and from the spectra of the two mutants A55U and A55C. The assignment of the peak at 6.30 ppm as belonging to A59 was made according to sequential G58 H1'-A59 H8-A59 H1' NOEs. After unambiguously identifying these four H1' resonances, the other sugar H1' protons (from G11, G56, A57 and G58) were assigned based on sequential NOE connectivities to the adjacent base, in conjunction with information from the G specific labeled sample and mutant RNAs. For example, the assignment of the A57 at 6.22 ppm was confirmed by the use of the mutant A57C. This mutant also facilitated the assignments of G56 and G58, because of slight changes in the chemical shifts effected by the mutation. The differentiation of G56 from G58 was made clear using the A55C and A55U mutants. Base and sugar resonances in G56 showed slight chemical shift changes in both mutants by the near-neighboring effect, whereas resonances in G58 did not.

The assigned sugar H1' protons were then used to correlate the remaining sugar protons. The H2' to H1' cross peaks of 4 nucleotides (G11, G56, A57 and G58) were initially identified in a COSY spectrum; this was possible because of the large $^{3}J_{HH}$ coupling constant of the C2'-endo
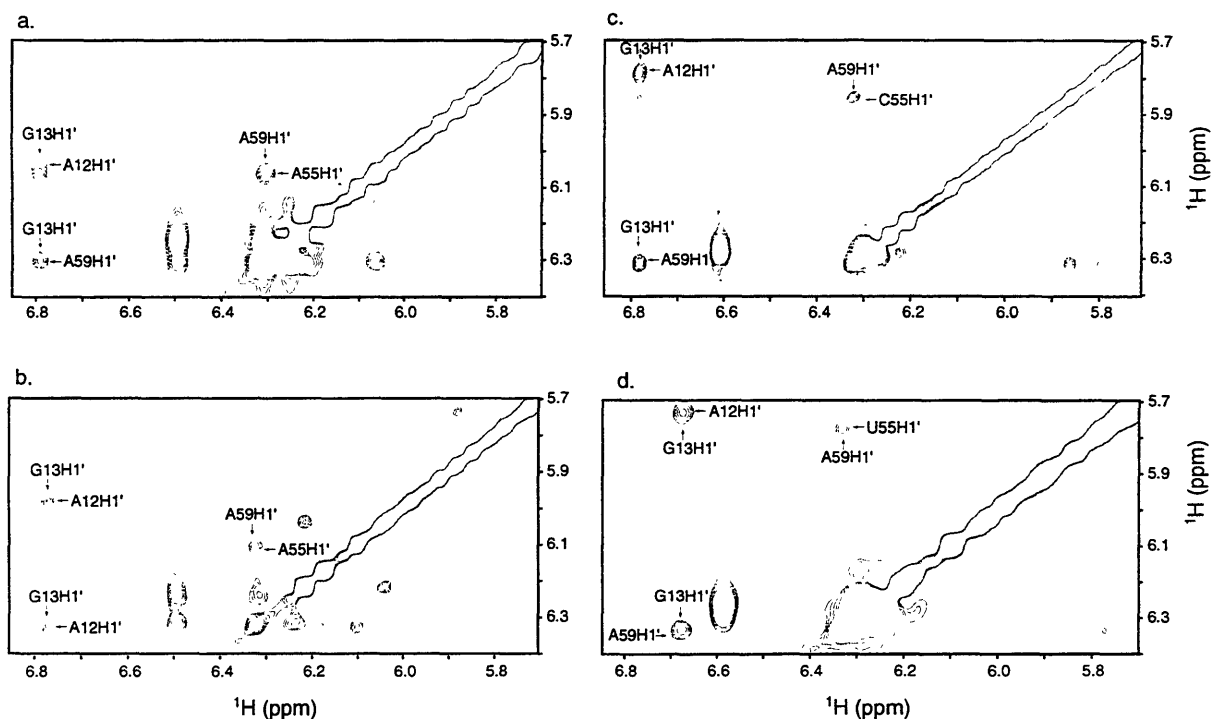
Figure 5.1.5: NOESY spectra showing sugar H1' regions of **a**, L30N (wt), **b**, A57C, **c**, A55C, and **d**, A55U RNAs in complexes with L30 protein. The existence of three sets of H1'-H1' sugar NOEs have been observed in all of these RNAs in the presence of protein. The mixing times used in the NOESY experiments are slightly different, with 50 ms in spectra **a** and **d**, and 150 ms for spectra **b** and **c**. Nevertheless, in each case, there are medium to medium strong NOEs between the G13 H1' and A12 H1', G13 H1' and A59 H1', and A59 H1' and A55 (or C55, U55) H1' protons.

sugar pucker. The assignments were subsequently cross-checked with a ^{1}H-^{13}C HSQC spectrum. It was essential to identify these H2' proton resonances because of the unusual chemical shifts adopted by these resonances. For example, the chemical shifts of the H1' and H2' of G56 are 4.64 ppm and 5.15 ppm, respectively, which the reverse order from what is expected. Another example is the H4' of A57; it has a chemical shift of 5.22 ppm, and could be mis-assigned to H2' proton.

The G56 was seen to exist in a different glycosidic conformation. This was concluded from the intensity of the G56 H8-G56 H1' cross peak which was much stronger than all the other aromatic to H1' cross peaks, and was comparable to the H5-H6 cross peaks of pyrimidines. In a short mixing time NOESY spectrum, the volume of this cross peak corresponded to a distance of approximately 2.5 Å, which is more than twice the volume of the G56 H8 to H2' cross peak. This indicates that the glycosidic torsion angle for G56 is in the *syn* conformation. As it was pointed out earlier in the imino proton assignment, there exists a strong G imino proton NOE to the G56 H8 resonance (7.38 ppm). The unusual G-G base pair hydrogen pattern and the glycosidic conformation indicate a possible role of G56 in the protein-RNA recognition.

More than 50% of the other sugar protons within the internal loop were assigned using ^{13}C-edited NOESY-HSQC and HCCH-TOCSY experiments, in conjunction with a homonuclear NOESY experiment. Nearly complete assignments were made to G11, G56, A57 and G58, supported by the unambiguous identification of the H2'/C2' which were in the C2'-endo conformation, and also by the information provided in the HCCH-TOCSY spectrum. On the contrary, only partial assignments were obtained for A12, A55, A59 and U60, as these resonances show exchange broadening. The broader linewidth of these resonances can be easily observed in different regions of the ^{13}C-edited HSQC spectrum. The peaks corresponding to these residues were either weak or absent in the HCCH-TOCSY spectrum making assignments ambiguous. Only partial assignments were made for the H2', H4', and only a few H3', H5' and H5" of these nucleotide were assignable using the NOE-based approach. The assignments involved the use of a short mixing time (50 ms) NOESY experiment, in which strong cross peaks were observed between H1'-H2' and H1'-H4' because of their close proximity (~2.5 to 3.0 Å and

~2.8 to 3.3 Å, respectively) (Wüthrich, 1986). Provided that there is no degeneracy in the H1' chemical shifts, the assignments of H2' and H4' were made, and they were differentiated using the ^{13}C-edited HSQC and ^{13}C-edited NOESY-HSQC correlation spectra.

The base and sugar proton and carbon chemical shifts thus obtained are listed in Table 5.1.

5.1.4. Torsion angles

5.1.4.1. Glycosidic torsion angles

The *anti* and *syn* glycosidic conformations for the residues of L30N RNA in the complex were obtained on the basis of the intensities of the NOE cross peaks between base (purine H8 or pyrimidine H6) protons and the H1' proton. A strong NOE is associated with a *syn* conformation (~ 2.5 Å distance), whereas weaker NOE indicates *anti* conformation (~ 3.7 Å distance) (Wüthrich, 1986). Among the nucleotides in both the helical stems and the internal loop region, only G56 in the internal loop exists in *syn* conformation. Consistent with the intensity of the H1'-H2' cross peak for this residue, the chemical shift of the H2' proton of G56 is shifted downfield to 5.15 ppm. This is a result of the in-plane ring current contribution associated with the *syn* alignment of purine bases and the C2'-endo sugar pucker in the complex. All other residues adopt the *anti* conformation with stronger base to H2' proton NOE patterns.

5.1.4.2. Sugar puckers

The sugar conformation (C3'-endo or C2'-endo) can be directly estimated from the $^{3}J_{H1'-H2'}$ coupling constants. A value greater than 10 Hz is indicative of C2'-endo conformation, whereas a value smaller than 2 Hz is indicative of C3'-endo conformation. Three bond $^{3}J_{H1'-H2'}$ coupling constants can be measured using homonuclear COSY and short mixing time (11 ms) TOCSY spectra. There were only seven H1'-H2' cross peaks clearly visible in both spectra, which correspond to G5, G11, C19, A20, G56, A57 and G58 (Figure 5.1.6). COSY spectrum generally

Table 5.1: Protein-bound L30N RNA proton, nitrogen and carbon chemical shifts. Proton chemical shifts are referenced to internal TSP. Nitrogen and carbon chemical shifts are referenced to external NH_4Cl and TSP, respectively. The errors in chemical shifts are ±0.02, 0.2, and 0.3 ppm for proton, nitrogen and carbon, respectively. Tentative assignments are indicated in ().

Residue	H1,H3/N1,N3	amino/amN	H8,H6/C8,C6	H2,H5/C2,C5	H1'/C1'	H2'/C2'	H3'/C3'	H4'/C4'	H5',H5"/C5'
G5			8.14/139.4		5.83/90.8	4.97/75.5	4.81/75.4	4.60/84.2	4.42,4.33/67.8
G6	12.54/146.8		7.66/137.6		5.94/93.1	4.69/75.9	4.32/74.0	4.64/83.2	4.37,4.51/67.4
A7			7.94/140.5	7.83/154.4	6.03/93.3	4.65/76.1	4.65/73.1	4.52/82.5	4.12,4.65/65.4
C8		8.38,6.99/97.2	7.34/140.6	5.26/97.7	5.34/94.2	4.29/76.0	4.20/73.0	4.42/82.3	4.06,4.47/65.6
C9		8.31,6.42/97.1	7.50/141.1	5.16/98.2	5.35/95.1	4.61/75.2	4.29/72.8	4.35/82.6	4.07,4.52/65.2
G10	10.87/146.0		7.52/136.2		5.51/93.2	3.43/76.1	4.45/73.0	4.22/83.3	4.45
G11	11.18/144.0		8.00/138.5		5.80/89.1	5.07/76.3	4.89/80.0	4.67/87.5	
A12			8.04/140.8	7.65/154.9	6.04/89.0	4.94/79.7	4.59/78.8	4.94/85.4	
G13	12.79/147.4	8.69/73.4	8.10/138.2		6.78/94.0	5.00/79.9	4.53/	4.99/83.8	4.38
U14	11.93/158.5		8.02/140.5	5.50/104.5	5.51/94.3	4.46/75.9	4.42/72.7	4.39/82.3	4.26,4.32/67.9
G15	12.93/147.4		7.92/137.7		5.88/93.3	4.57/75.7	4.59/73.6	4.58/82.7	4.36,4.16/67.4
U16	14.22/162.4		7.78/142.3	5.17/103.0	5.59/94.5	4.48/76.0	4.43/73.1	4.44/82.3	4.15,4.54/65.3
C17		8.19,6.73/97.9	7.67/141.2	5.56/98.4	5.60/94.7	4.30/76.0	4.50/72.6	4.42/72.4	4.11,4.57/64.9
G18	10.94/146.0		7.78/137.2		5.63/92.4	4.57/76.7	4.69/74.9	4.44/83.2	4.43,4.12/65.9
C19		7.11/97.1	7.77/144.5	5.72/98.6	5.68/92.0	4.38/76.4	4.32/76.0	4.15/84.2	4.15,3.98/66.6
A20			8.01/141.1	7.92/155.0	5.63/91.7	4.38/77.5	4.57/76.6	4.22/84.4	3.77,3.88/66.9
A21			8.27/142.8	8.03/155.3	6.10/97.8	4.77/77.4	5.43/75.5	4.55/83.6	4.44,4.53/67.4
G22	12.35/146.6			7.82/137.9	4.07/92.9	4.42/75.4	4.30/74.8	4.40/83.3	4.58,4.22/66.9
A51			7.79/139.9	7.79/154.2	6.02/93.3	4.50/76.1	4.64/72.7	4.50/82.6	4.13,4.56/64.9
C52		8.55,6.99/98.3	7.42/141.0	5.22/97.8	5.51/93.6	4.41/76.4	4.40/73.1	4.41/82.1	4.07,4.51/65.3
G53	11.14/143.9	6.35/72.9		7.53/137.6	5.80/94.0	4.65/75.6	4.28/74.1	4.52/83.1	4.08,4.41/64.8
C54		7.97,6.99/98.8	7.55/140.5	5.23/97.7	5.60/94.2	4.47/75.7	4.52/72.0	4.47/82.5	4.11,4.52/64.8
A55			8.18/140.2	8.14/155.3	6.03/91.0	4.03/76.3	4.65		4.20/87.8
G56	8.98/146.0			7.38/140.8	4.63/90.8	5.14/71.5	4.45/79.2	3.44/85.5	3.81,4.04/69.0
A57			8.67/143.1	8.32/156.4	6.24/89.7	4.63/80.1	4.60/78.3	5.24/85.4	4.32,4.04
G58			8.40/139.0		6.17/86.1	5.14/77.5	5.08/82.1	4.88/87.2	4.48,4.31
A59			8.25/143.7	7.65/154.9	6.30/94.6	4.90	4.62	4.82/81.7	4.26
U60	11.79/158.4		7.35/142.5	5.77/105.5	5.73/93.0	4.53		4.40	4.36
G61	12.29/146.7		7.74/137.2		5.52/94.2	4.31/75.8	4.55/74.0	4.50/83.0	4.30/68.6
G62	13.28/148.2		7.21/136.7		5.81/93.6	4.53/75.8	4.56/72.9	4.51/82.6	4.57,4.11/65.1
U63	14.22/162.5		7.66/142.3	5.10/102.9	5.58/94.3	4.57/75.6	4.51/72.7	4.46/82.2	4.13,4.44/65.3
C64		8.64,7.13/98.2	7.84/142.1	5.69/98.1	5.60/94.3	4.40/76.8	4.68/74.5	4.48/83.0	4.15,4.52/64.6
C65			7.70/143.5	5.52/98.7	5.77/93.1	4.03/77.9	4.20/70.2	4.20/83.8	4.06,4.54/65.6
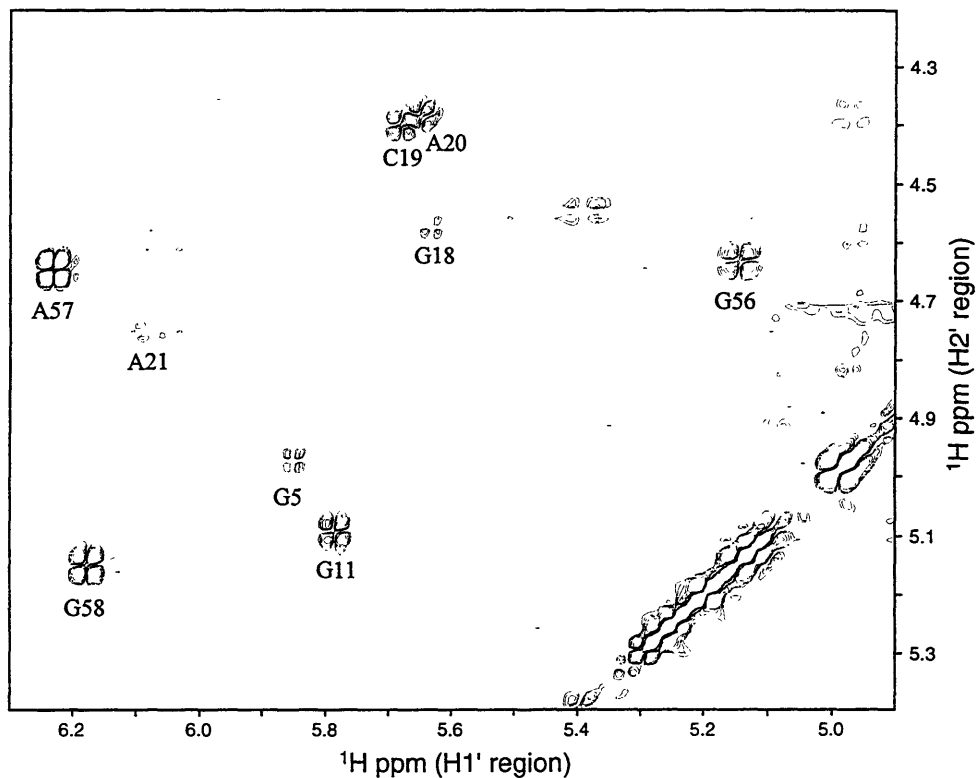
Figure 5.1.6: Ribose H1' and H2' proton regions of a DQF-COSY spectrum for the L30N RNA in the bound form. The plotted horizontal axis and vertical axis represent H1' and H2' proton chemical shifts, respectively, except for G56 which has the reverse orientation. Peaks are labeled with the assignments. The large H1' and H2' coupling constants (up to 12 Hz) suggest unusual sugar pucker conformations (*i.e.*, C2'-endo) for these residues .

leads to overestimation of the *J*-couplings, as there are partial cancellation of positive and negative peaks due to line broadening effects. Nevertheless, rough estimates could be made for these resonances. All the peaks in the present case correspond to *J*-couplings greater than 5 Hz, suggesting a possible C2'-endo or inter-conversion between C2'-endo and C3'-endo sugar conformations. The $J_{H1'-H2'}$-couplings for nucleotides G5 at the 5'-terminal, and C19 and A20 at the tetraloop are expected to be greater than 5 Hz. The sugar puckers of these types of nucleotides have been reported to have approximately 60% C2'-endo conformations (Heus & Pardi, 1991b). In particular, G11, G56, A57 and G58 have splittings greater than 8 Hz, suggesting these residues adopt primarily C2'-endo pucker conformation. The C2'-endo type of sugar puckers has been reported to be important in expanding the sugar-phosphate backbone in the loop, therefore suggesting unique conformations adopted by these nucleotides in the complex. In contrast, the nucleotides in the C2'-endo conformation, H1'-H2' peaks of other nucleotides in L30N RNA were not observed in either spectrum, which is expected if the sugars are in the C3'-endo conformation.

Table 5.2 shows the summary of all the experiments performed on the L30N RNA in the complex.

5.2. Unusual Features in the RNA NMR Spectrum

After obtaining nearly complete assignments for the RNA in the complex, the chemical shifts of the RNA resonances and the NOESY spectra were analyzed more vigorously to identify various unusual features that are direct signatures of the unusual conformation of the individual residues. A systematic description of all these features is discussed below.

Table 5.2: Data acquisition parameters and pulse sequences used in NMR experiments on L30N RNA in complex. Unless specified, all experiments were acquired at 30°C. The four subdivisions in each NMR dimension (*F1*, *F2*, or *F3*) are: **a**, complex data points, **b**, spectral width (Hz), **c**, carrier position (ppm) and **d**, nuclei. In a 2D experiment, *F2* is the direct acquisition dimension, whereas *F1* is the indirect detection dimension. In a 3D experiment, *F3* is the direct acquisition dimension, whereas *F1* and F2 are the indirect detection dimensions.

Molecule	Experiment	*F3*(a, b, c, d)	*F2*(a, b, c, d)	*F1*(a, b, c, d)	T°C	Mixing time (ms)	Reference
L32 Protein /L32N RNA	NOESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H	15, 25, 30, 35	50, 100, 150, 300	
	NOESY-watergate (Bitter 501)	-	2048, 11990, 4.73, ^{1}H	512, 11990, 4.73, ^{1}H	15, 25, 30	100, 150	
	NOESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H	15, 30	100	
	dq-COSY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H			(Rance *et al.*, 1983)
	ROESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H		50	(Bax & Davis, 1985)
L32(U$_{14}$C) RNA/ ^{15}N L32 Protein	TOCSY (Bitter 501)	-	2048, 60002.4, 4.73, ^{1}H	512, 6002.4, 4.73, ^{1}H		11	(Brauschweiler & Ernst, 1983)
	NOESY (Bitter 750)	-	2048, 9000, 4.73, ^{1}H	512, 9000, 4.73, ^{1}H	25	400	
L32(A$_{57}$C) RNA/L32 Protein	NOESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H		150, 300	
	NOESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H		100	
	dq-COSY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H			(Rance *et al.*, 1983)

Sample	Experiment					Reference
L32(A$_{55}$C) RNA/ ^{15}N L32 Protein	NOESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H	150, 300	
	NOESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H	100	
	dq-COSY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H		(Rance et al., 1983)
L32(A$_{55}$U) RNA/ ^{15}N L32 Protein	NOESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H	150, 300	
	NOESY-watergate (Varian)	-	2048, 14000, 4.73, ^{1}H	512, 14000, 4.73, ^{1}H	100	
	TOCSY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H		(Brauschweiler & Ernst, 1983)
	ROESY (Varain 600)	-	2048, 6600, 4.73, ^{1}H	512, 6600, 4.73, ^{1}H	50	(Bax & Davis, 1985)
L32 protein / ^{15}N, ^{13}C L32N RNA	^{15}N FHSQC (Bitter 501)	-	1024, 11000, 4.73, ^{1}H	128, 5000.0, 117.91, ^{15}N		(Mori et al., 1995)
	^{13}C FHSQC (Varian 600)	-	1024, 7000, 4.73, ^{1}H	128, 14000, 116.17, ^{13}C		(Mori et al., 1995)
	^{13}C HSQC (Varian 600)	-	1024, 6000, 4.73, ^{1}H	128, 7000, 82.97, ^{13}C		(Bax et al., 1990)
	^{13}C HSQC-CT (Varian 600)	-	1024, 6000, 4.78, ^{1}H	200, 7000, 82.97, ^{13}C	25	(Santoro & King, 1992)
	^{13}C HSQC-NOESY (Varian 750)	512, 6000, 4.78, ^{1}H	128, 6000, 4.73, ^{1}H	32, 4000, 82.97, ^{13}C	150	(Clore et al., 1990)
	^{13}C HSQC-NOESY arom (Varian 600)	512, 6000, 4.78, ^{1}H	128, 6000, 4.73, ^{1}H	32, 4000, 140, ^{13}C	100	(Clore et al., 1990)

	^{13}C HCCH-TOCSY (Varain 600)	512, 5000.0, 4.78, ^{1}H	128, 3000.0, 4.73, ^{1}H	32, 4000, 82.97, ^{13}C		12	(Kay et al., 1993)
	^{15}N,^{13}C HSQC-NOESY (Varian 600)	512, 6600, 9.00, ^{1}H	96, 6600, 9.00, ^{1}H	22, 4000, 119.58, ^{13}C (^{15}N)		150	(Pascal et al., 1994)
	^{15}N NOESY-HMQC (Varian 600)	1024, 18000, 4.73, ^{1}H	128, 18000, 4.73, ^{1}H	16, 4000, 119.58, ^{15}N		100	
L32 protein/ ^{15}N,^{13}C G only L32N RNA	^{15}N FHSQC (Bitter 501)	-	1024, 11000, 4.91, ^{1}H	128, 5000.0, 117.91, ^{15}N	15		(Mori et al., 1995)
	^{13}C FHSQC (Varian 600)	-	1024, 7000, 4.87, ^{1}H	128, 14000, 116.17, ^{13}C	15, 25, 30		(Mori et al., 1995)
	^{13}C HSQC (Varian 600)	-	1024, 6000, 4.78, ^{1}H	128, 7000, 82.97, ^{13}C	25		(Bax et al., 1990)
	^{13}C HSQC-CT (Varian 600)	-	1024, 6000, 4.78, ^{1}H	200, 7000, 82.97, ^{13}C	25		(Santoro & King, 1992)

5.2.1. Unusual sugar carbon chemical shifts

Recent studies have showed that the sugar C1', C3' and C4' carbons exhibit distinct chemical shifts for sugars in C2'-endo or C3'-endo conformations with *anti* glycosidic torsion angles. More specifically, while the C1' carbons resonate at ~86 ppm for C2'-endo sugar pucker conformations, they are shifted downfield by about 7 ppm for C3'-endo sugar puckers. In contrast, the C3' carbons resonate at ~78 ppm and C4' carbons at ~86 ppm for C2'-endo sugars, and both are shift upfield by ~5 ppm for C3'-endo sugar puckers (Greene *et al.*, 1995; Greenbaum *et al.*, 1995; Jiang *et al.*, 1996). According to the above empirical criteria, the chemical shifts (C1', C3' and C4') of G11 (89.1, 78.3 and 87.4 ppm, respectively), G56 (90.7, 79.2 and 85.4 ppm), A57 (89.7, 78.3 and 85.4 ppm), and G58 (86.0, 82.1 and 86.5 ppm) in the L30N RNA were identified to be in C2'-endo sugar conformations. This is in agreement with the observation of large $^3J_{H1'-H2'}$ coupling constants in the COSY spectrum.

5.2.2. Unusual intra- and inter-molecular NOEs

5.2.2.1. Unusual NOEs within the internal loop

Conformation of G11: The G10 H1' and H2' sugar protons show weak connectivities to the G11 base proton, and this suggests that the base stacking is poor in this step and an extension of the phosphate occurred this step. On the contrary, there are strong NOE cross peaks for the G11 H1' to A12 H8, and G11 imino and amino protons to the base and sugar protons of A12 (H2, H1' and H2' protons), suggesting the stacking of the G11 above A12. Weak intraresidue sugar to base cross peaks, weak sequential NOEs for A12, together with medium strong NOEs between sugar H1'-H1' imply an unusual turn at the G11 to A12 positions. In addition, the observation of a strong NOE between the imino-proton of G11 and the H8 proton of G56 appears to be consistent with the formation of a G11-G56 base pair involving the Watson-Crick edge of G11 and the reverse Hoogsteen edge of G56 in the complex.

The sequential base-H1' protons exhibit a very weak NOE at the G10-G11 step. Although there is a medium NOE between the G10 H2' and the G11 H8, the chemical shift of the H2' has been shifted upfield to 3.44 ppm, which is most likely due to ring current effects. It appears from the NOE patterns that the G11 makes a kink in the sugar-phosphate backbone. Specifically, the G11 H2' and H3' protons display only strong NOEs to its own base H8 proton, but no inter-residue NOEs to either the 3'- or 5'-flanking bases. On the contrary, the H1' proton of G11 exhibits strong NOE to the base H8 of A12. This can be explained by the C2'-endo conformation of the G11 residue which displays the H2' and H3' protons away from the backbone. Furthermore, G11 is involved in the formation of a G11-G56 mismatch, involving the imino proton of G11 and the H8 proton of G56. Spin diffusion peaks from the imino proton of G11 to the H1' of G56 were observed. These observations suggest that G11 and G56 undergo hydrogen bonding involving the imino proton of G11 and the N7 of G56, and an amino proton of G11 and the O6 of G56.

Position of A12: Though the NOEs between A12 H1'-G13 H8 and A12 H2'-G13 H8 protons are relatively weak, the A12 H1'-G13 H1' protons show a very strong NOE. Furthermore, A12 did not exhibit any stacking pattern to the G13-C54 pair, as there are no NOEs observed from either A12 H2 or H8 to the G13 imino proton. The medium-strong NOE observed between H2 of A12 to H2 of A55 suggests that these two base pairs are close in space, though no direct evidence can be obtained for the formation of an A12·A55 base pair. However, indirect evidence involving the A55U mutant suggests there is no base pair between residues 12 and 55, since no base pairing NOE pattern is observed between A12 and U55.

Stacking of A55 below the G13-C54 pair: There are at least three separate observations that suggest the complete stacking of A55 below the G13-C54 pair. First, there is a medium C54 H1' to A55 H8 NOE, and strong C54 H2'-A55 H8 and C54 H3'-A55 H8 NOEs. Second, in a long mixing time NOESY (300 ms), there are base proton stacking NOEs between A55 H8 and C54 H5, H6. Moreover, there is a weak NOE between the A55 H2 and G13 imino protons, which

disappears upon mutation of A55 into either U or C. All these experimental data indicate that A55 is fully stacked below G13-C54 pair.

Conformation of G56: The G56 base with its *syn* glycosidic torsion angle and its C2'-endo sugar pucker displays no sequential base to sugar NOEs to the adjacent A55, and very few weak sugar to base NOEs to A57. Compared to other sugar protons in G56, H4' displays a number of interbase NOEs, to base and sugar protons of A57, and further extends to base and sugar protons of G58. An unusual large upfield chemical shift for H4' (3.4 ppm) of G56 further supports the proposed positioning of G56. It appears that the NOE patterns require the G56 and A57 bases to be orthogonal to each other so that the A57 base is positioned along and over the C4'-C5' backbone of G56. Also, unusual NOEs are observed between the H8 proton of A57 and the H4', H5'/H5" protons of G56. This alignment is facilitated by both G56 and A57 adopting predominantly C2'-endo sugar pucker conformations in the complex, therefore extend the RNA backbone at this segment. The observed strong NOE between the imino proton of G11 to the H8 proton of G56 appears to be consistent with the formation of a G11-G56 mismatch involving the Watson-Crick edge of G11 and the reverse Hoogsteen face of G56 in the complex.

There are several unusual NOEs within the G56, A57 and G58 bases that are suggestive of a hairpin-like chain reversal by the segment. The extremely weak sequential NOE between G56 and A57 suggests that the chain reversal must occur at this step. The weak to almost absent sequential NOEs between A57 and G58, together with the C2'-endo sugar pucker adopted by both residues, implies an extended backbone at the A57 and G58 step.

Conformation of A57: Upon protein binding, the resonances of A57 display much sharper linewidths than the rest of the RNA. The near absence of NOEs to any adjacent sugar protons from either aromatic H8 or H2 protons of A57 implies that the base is loop out of the RNA helix.

Conformation of G58: Although there is a weak NOE from the G58 H8 to A57 H8 proton in the long mixing time NOE (through spin diffusion), the stacking of the two bases is not extensive. This is interpreted as there is only a weak H1'-base NOE from A57 to G58. The H2' and H3'

protons of A57 also display weak connectivities to G58 base protons. In contrast, the H4' proton of A57 displays a medium NOE to G58 base proton. The presence of a G56 H4' NOE to the G58 H8 proton suggests a sharp kink in the backbone from the G56-A57-G58 region.

Conformation of A59: The sequential base-sugar H1' NOE connectivity resumes at the G58-A59 step, indicative of the continuation of the right-handed stacking alignment from the A59 residue. But the presence of a medium strong long range NOE from the sugar H1' proton of A59 to the H1' proton of A55, and a medium long-range NOE from the H1' of A59 to the H1' proton of G13 suggest a distortion at the phosphate backbone of A59. In addition, the H1' proton of A59 also exhibit medium NOEs to H2 protons of A12 and A55. The unusual NOE patterns put A59 in a conformation that appears to be perpendicular to G13 and parallel to the G11-G56 base pair.

Extension of the helical stems into the internal loop: The stacking of the internal loop with adjacent Watson-Crick helical stem segments represents the unusual conformation adopted by the asymmetric internal loop upon protein binding. The NOE and chemical shift patterns indicate that the leftward stem is extended through stacking of the G11 (*anti*) - G56 (*syn*) mismatch pair to the G10-U60 wobble base pair in one direction and only partial stacking of G11 with G10. On the contrary, the rightward stem stacking is only extended through A55 to the Watson-Crick G13-C54 pair in one direction, is broken between G56 to G58, and resumes at A59 with partial stacking to U60 of the G10-U60 wobble pair.

5.2.2.2. Long range NOEs between the RNA and protein

Long range NOEs between RNA and protein have been observed in all NOESY experiments acquired on either nonlabeled or ^{13}C/^{15}N-labeled samples. Detailed studies of these intermolecular contacts were carried out by the analysis of homonuclear NOESY spectra acquired in H_2O and D_2O in combination with heteronuclear NOESY spectra of labeled molecule with its nonlabeled counterpart. More specifically, these are ^{15}N-edited NOESY-HSQC spectra of ^{15}N-labeled L30 protein/nonlabeled L30N RNA, ^{15}N-edited NOESY-HSQC spectra of ^{13}C/^{15}N-labeled L30N RNA/nonlabeled L30 protein, ^{13}C-edited NOESY-HSQC spectra of ^{13}C/^{15}N-labeled

L30 protein/nonlabeled L30N RNA, ^{13}C-edited NOESY-HSQC spectra of ^{13}C/^{15}N-labeled L30N RNA/nonlabeled L30 protein. All ^{15}N-edited NOESY spectra were acquired in H_2O, whereas ^{13}C-edited NOESY spectra were recorded in both H_2O and D_2O.

The most distinct long-range NOEs that can be observed in homonuclear experiments originate from two types of resonances. The first types were observed in H_2O, where the downfield shifted imino-resonances in the RNA (between 10 and 14.5 ppm) are isolated from the protein exchangeable resonances. The second types were observed in D_2O, from the aromatic and some H1' resonances that are separated from most protein proton resonances, since the labile amide protons from either the protein or the RNA are exchanged away by the solvent. In both categories, direct RNA-protein NOEs were observed through the downfield RNA proton resonances to the upfield proton resonances to the protein side chains (Figure 5.2.1). Other RNA resonances in close proximity of the protein were observed in the ^{13}C-edited NOESY-HSQC experiment. The results of the intermolecular NOEs are summarized in Table 5.3.

Unusual long range intermolecular NOEs have been observed for many residues within the internal loop, including G11, A55, G56, A57 and G58, but most of them were located around residues 55 to 58 on one side of the internal loop. Among the nucleotides that are in close proximity to the protein, G56 gives rise to most of the distinct long range NOEs to the protein residues. The frequently observed resonance is the H8 of G56, which gives NOEs to backbone amides, nonexchangeable side chain and aromatic protons of the protein as seen in various 2D and 3D NOESY spectra. The contacting protein residues include Leu25, Gly25, Tyr27, Phe85 and Val87, many of which being hydrophobic residues.

In summary, the comprehensive assignments of the aromatic and ribose ^{1}H, ^{13}C and ^{15}N resonances of the L30N RNA in the bound form were made through analysis of homonuclear and heteronuclear experiments. Studies of the spectra with ^{13}C/^{15}N-G-specific labeled RNA and homonuclear spectra of three RNA mutants also facilitate the unambiguous resonance assignments, thus allowing identification of many unusual long range intra-molecular NOEs within the internal loop and inter-molecular NOEs between the RNA and the protein. The G11-
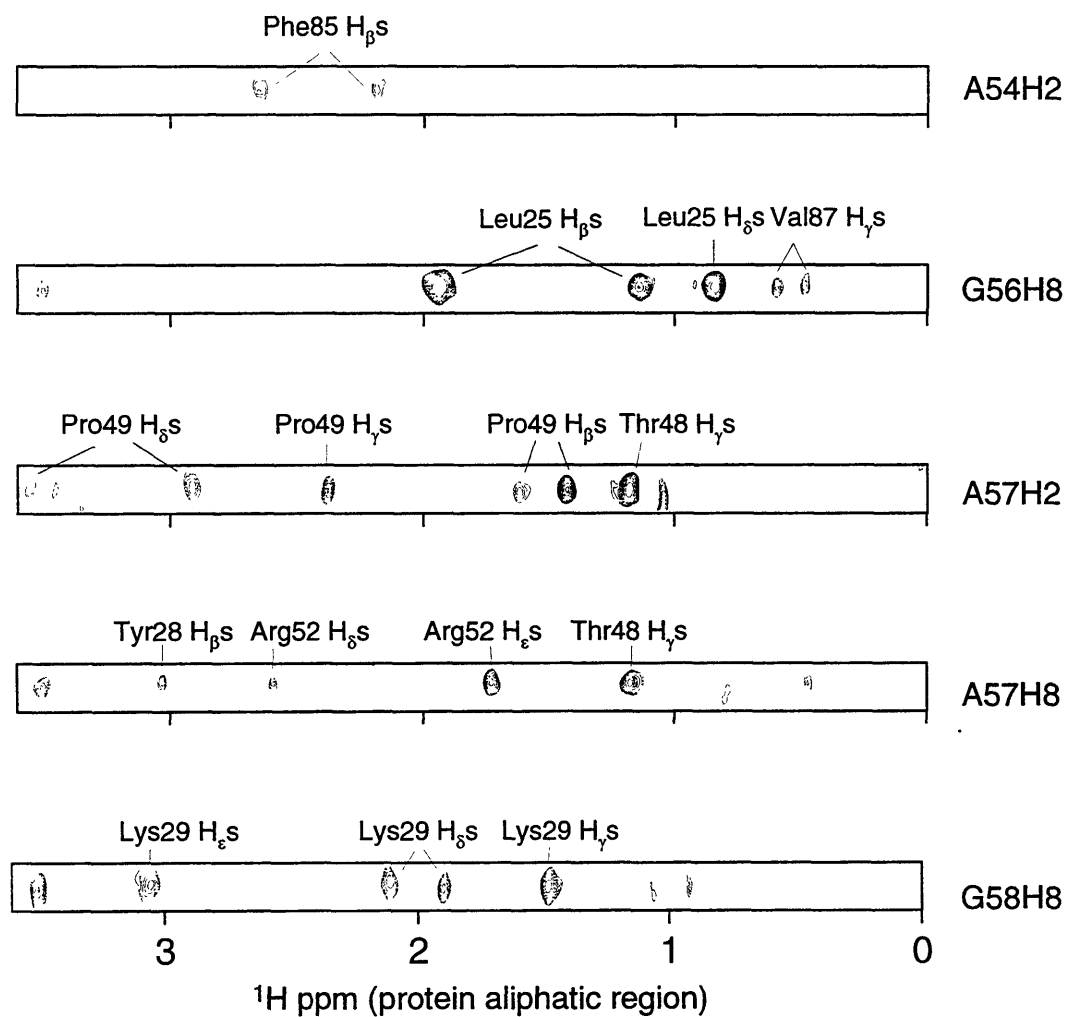
Figure 5.2.1: Unusual intermolecular NOEs identified from a NOESY spectrum recorded in D_2O. Assignments for the RNA bases are given in Table 5.1.

Table 5.3: Unusual long-range inter-molecular NOEs. For simplicity, NOEs are group together by the order of RNA residues. For a more detailed list, see **Appendix C** for the entire NMR restraint set for molecular modeling.

RNA	Protein	NOE intensity
G11 (H1)	Ser26 (H_N)	very weak
G11 (H1)	Ser29 (H_N, β)	very weak
A55 (H2)	Phe85 (β, δ, ε, ζ)	very weak - medium
G56 (H1', 2', 3', H8)	Leu25 (H_N, α, β, γ, δ)	very weak - medium
G56 (H1', H8)	Gly26 (H_N, α)	weak, medium
G56 (H2', 3', H1, H8)	Phe85 (β, δ, ε, ζ)	very weak - medium
G56 (H8)	Tyr27 (H_N)	very weak
G56 (H8)	Val87 (H_N, α, β, γ)	very weak
G56 (H8)	Gly88 (H_N, α)	very weak
A57 (H1', H8)	Arg52 (γ, δ, ε)	very weak - medium
A57 (H4')	Lys28 (H_N)	very weak
A57 (H4', 5',")	Tyr27 (H_N, α, β, δ, ε)	very weak - medium
A57 (H2)	Thr48 (H_N, α)	very weak
A57 (H2)	Pro49 (α, β, γ)	very weak - medium
G58 (H1', 2', H8)	Tyr27 (H_N, α, β, δ, ε)	very weak - weak
G58 (H1', 2', H8)	Lys28 (H_N, α, β, γ, δ, ε)	very weak - weak
G58 (H2', H8)	Arg52 (γ, δ, ε)	very weak - medium
G58 (H4')	Ser29 (H_N, β)	very weak

G56 the reverse Hoogsteen base pair was identified, of which G56 adopts an unusual *syn* glycosidic conformation. The phosphate backbone from G56 to G58 seems to undergo a dramatic kink at this region, with the A57 and the G58 stacking away from the main RNA helix. The existence of three pairs of long-range H1'-H1' NOEs between A12 and G13, G13 and A57, and A55 and A57 further supports the distortion of the phosphate backbone. Studies of the ^{13}C chemical shifts and the torsion angle measurements facilitated the identification of the unusual C2'-endo conformation of the four RNA residues (G11, G56, A57 and G58) within the internal loop. These unusual conformations are facilitated by the complex formation, which are stabilized either by hydrogen bonding between nucleotides or interactions with protein residues. Among those interacting with the protein, residues on one side of the loop (A55, G56, A57 and G58) seem to make extensive contacts with proteins. These unusual RNA conformations and RNA-protein interactions will become obvious from the structural modeling, which will be discussed in the next two chapters.

Chapter 6. Molecular Modeling

The previous chapters described the substantial efforts, spanning the fast few years, recording many NMR experiments and analyzing many spectra to obtain comprehensive sequence-specific assignments of the L30 protein and RNA. While these assignments usually do not reveal much about the tertiary structure of the complex, they are a prerequisite for achieving the biochemical goal. This ultimate purpose is to obtain the structure of the L30 protein-RNA complex and to understand the functional recognition elements within the complex. With these sequence-specific assignments in hand, the next step towards the ultimate goal is to solve the three-dimensional structure of the L30 complex through molecular modeling.

There are currently four types of information obtained by NMR analyses that can be useful in providing restraints for generating structures: dihedral angles and interproton distances (Wüthrich, 1986), chemical shifts (Kuszewski *et al.*, 1995) and dipolar vector orientations (Tjandra & Bax, 1997). Dihedral angles are derived from measuring scalar coupling constants, interproton distances are interpreted from the nuclear Overhauser effect (NOE), chemical shifts are themselves manifested through sequence-specific assignment of resonances, and dipolar vectors in an oriented molecule are derived from residual dipolar coupling constants. Currently, the first two categories of information, namely dihedral angles and interproton distances, constitute the main stream in providing NMR-derived restraints for macromolecular structure determination. The other have started to be implemented into structure refinements in recent couple of years, although their application has not completely matured. Hence the latter two (chemical shifts and dipolar vectors) have not been fully explored as restraints for generating L30 complex structure haven't been fully explored during the course of this thesis work.

In the modeling process, the coupling constants and NOE information obtained for the L30 protein and RNA in the complex are subsequently converted into respective dihedral angles and distance restraints, which can be used in either distance geometry (DG) or restrained molecular dynamics (rMD) structure calculations. Distance geometry procedures determine an ensemble of 3D structures solely on the basis of the consistency of distance constraints without

the involvement of dihedral angles (Crippen & Havel, 1988). The restrained molecular dynamics approach provides an alternative to the distance geometry methods by involving both distance and dihedral as part of the molecular force field. In addition, the structural potential of the target is reduced through temperature heat-cool annealing cycles (Nilges *et al.*, 1991). Typically, a hybrid method has been used in many laboratories to generate protein structure, in which initial structures are generated in distance geometry and later refined with restrained molecular dynamics (Nilges *et al.*, 1988). In the case of the L30 complex, both DG and rMD approaches were tested initially for generating the protein and RNA structure; only the restrained molecular dynamics gave better folded protein and RNA structures. Therefore, the subsequent calculations were carried out using mainly rMD, which will be discussed in detail in the following sections. These calculations were performed using the X-PLOR program (Brünger, 1992). Figure 6.0.1 and 6.0.2 illustrate the overall schemes for NMR-derived structure determination son the initial protein and RNA structures in the complex.

6.1. NMR-derived Structural Restraints

6.1.1. Theory

Currently, interproton distances and dihedral angles are two commonly experimentally-derived restraints used in generating an ensemble of NMR derived structures. The distances can be calculated from NOE intensities measured in NOESY experiments, since the cross relaxation rate constant of two spins is inversely proportional to the sixth power of their distance:

$$\sigma_{NOE} \propto \{-1 + 6/(1+4\omega^2\tau_c^2)\}/r^6$$

where ω is the spectrometer frequency, τ_c is the correlation time and r is the distance between the two spins. The NOE intensity between two dipolar-coupled spins is related to the cross relaxation rate constant and the time to which it occurs:

```
┌──────────────────────────────────────┐                    ┌─────────────────────────┐
│         Resonance assignment          │                    │    Identification of    │
│                                        │                    │       secondary         │
│  (a) Sequential backbone:  3D ¹⁵N-edited│ ─────────────────▶ │  structure elements     │
│  NOESY and TOCSY, 3D triple resonance  │                    └─────────────────────────┘
│  HNCA, HNCO, HN(CO)CA, HNCACB          │
│                                        │
│  (b) Side chain:  3D HCCH-TOCSY        │
└──────────────────────────────────────┘
```

(a) **Sequential backbone:** 3D ^{15}N-edited NOESY and TOCSY, 3D triple resonance HNCA, HNCO, HN(CO)CA, HNCACB

(b) **Side chain:** 3D HCCH-TOCSY

Iterative Cycle

Stereospecific assignment and torsion angle determination

(a) **Coupling constants:** 2D COSY, ^{1}H-^{15}N HMQC-J, 3D ^{1}H-^{15}N HNHA

(b) **Intraresidue and sequential distance restraints:** 3D ^{15}N-edited NOESY, 3D ^{13}C-edited NOESY

Simulated annealing

(a) **Random SA**

(b) **SA-regularization**

(c) **SA refinement**

Tertiary long-range distance restraints

3D ^{15}N-edited NOESY, ^{13}C-edited NOESY

Initial ensemble of protein structures

Iterative Cycle

Figure 6.0.1: Outline of the strategy used to generate the initial L30 protein structure in the complex.
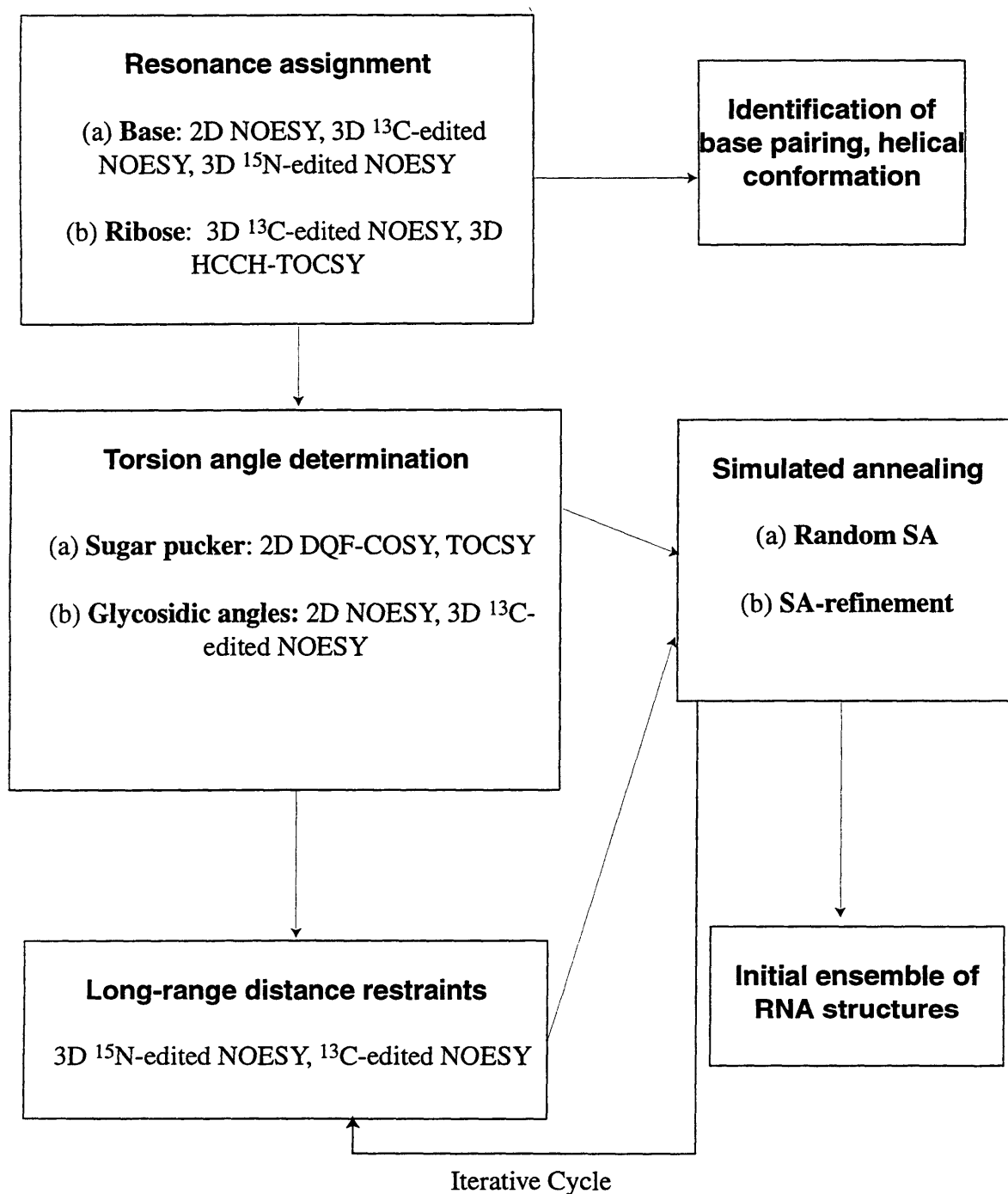
Figure 6.0.2: Outline of the strategy used to generate the initial L30 RNA structure in the complex.

$$I_{NOE} \propto \sigma_{NOE}/\rho$$

where $1/\rho$ is the duration of cross relaxation period. In a fixed mixing time, an unknown distance r_{ij} between two dipolar-coupled protons can be determined from a reference distance r_{kl} based on their NOE intensities

$$r_{ij} = r_{kl} \, (I_{kl} / I_{ij})^{1/6} \tag{6.1}$$

where I_{kl} and I_{ij} are respective NOE intensities.

Contrary to interproton distance derivations, dihedral angles are directly calculated from *J*-coupling constants measured in COSY-type experiments using the Karplus equation (Karplus, 1963), and in some cases with the combination of stereo-specific assignments in proteins. For instance, the backbone dihedral angle ϕ is calculated from the Karplus equation:

$$J(\phi) = A \cos^2(\phi - 60) + B \cos(\phi - 60) + C \tag{6.2}$$

where $A = 6.51$, $B = -1.76$, $C = 1.60$ (Vuister & Bax, 1993).

6.1.2. Protein restraints

6.1.2.1. Distance restraints

Three types of NOESY experiments were used to obtain NOE-based distance restraints. 3D ^{15}N-edited NOESY-HSQC spectra were used to derive NOEs originating from backbone and side chain exchangeable protons; 3D ^{13}C-edited NOESY-HSQC spectra were used to derive NOEs originating from nonexchangeable protons (*i.e.*, aliphatic side chains); and 2D homonuclear NOESY spectra were mainly used to derive NOEs originating from aromatic side chains. Distances derived in a 2D homonuclear NOESY spectrum utilize the known distance of

a Tyr H_δ/H_ϵ (2.4 Å) cross peak as a reference distance to calibrate the other distances. However, this reference information is lost in a 3D NOESY spectrum. In the 3D experiments, NOE cross peak intensities are not only determined from the cross-relaxation between two dipolar-coupled spins, they are also affected by the T_2 relaxation of the attached heteronucleus. The differences in NOE absolute intensities are particularly obvious by comparison of those from the terminal residues to those in the protein core. Even within the same residue, an NOE intensity originating from an H_α, say to a methyl proton, is different from that of the same methyl proton to the same H_α. Therefore, the NOE cross peak to diagonal ratio was used to determine distances in a 3D NOESY-HSQC spectrum.

Because of complications from cross-relaxation by spin-diffusion, the intensities of NOE cross peaks were classified as strong, medium, weak and very weak, corresponding to upper distance bounds of 3.0, 4.0, 5.0 and 6.0 Å, respectively , and the same lower bound limit set to 1.8 Å. 1.0 Å was added to the upper distance limits for NOEs involving methyl protons to account for the higher apparent intensity of methyl resonances. Distances involving methyl groups and non-stereospecifically assigned methylene protons were represented as pseudoatoms.

In addition to NOE-based distance restraints, hydrogen bonds were also supplemented in backbone distance restraints. The hydrogen-bonded amide protons were identified from D_2O exchange experiments with slow solvent-exchange rates, in combination with the presence of characteristic NOE patterns of regular secondary structure. Each hydrogen bond was represented by two distance restraints (2.95 Å for N to O and 1.95 Å for HN to O) to preserve linear bond geometry.

6.1.2.2. Dihedral angles

Dihedral angles of backbone and side chain atoms were restrained based on observed scalar coupling constants. In addition, stereospecific assignments are necessary for the side chains with prochiral centers. Within ϕ and ψ backbone dihedral angles of each residue, only ϕ angles have been determined. The ϕ dihedral angle restraints were derived from $^3J_{HNHA}$ coupling

constants determined from an HNHA experiment, with the addition of uncertainty boundaries. In most cases, where $^3J_{HNHA} < 5.5$ Hz, the backbone dihedral angle ϕ was restrained to $-65° \pm 25°$; when $^3J_{HNHA} > 8.0$ Hz, the backbone dihedral angle ϕ was restrained to $-120° \pm 40°$. Among the side chain dihedral angles of each residue, only χ_1 angles have been determined using $^3J_{H\alpha H\beta}$ coupling constants and, if necessary, stereospecific assignments of β-methylene protons. The resulting χ_1 angle was correspondingly assigned to one of the three rotamers: $+60°$, $+180°$ and $-60°$. The stereospecific assignments of two-methyl groups at the prochiral center of Val residues were achieved only after completion of the initial calculations.

6.1.3. RNA restraints

6.1.3.1. Distance restraints

Similar to those in L30 protein, 3D ^{13}C-edited NOESY-HSQC, 3D ^{15}N-edited NOESY-HSQC, and a series of 2D homonuclear NOESY experiments were used to generate distance restraints for the RNA. Interproton distances between nonexchangeable base to H1' protons were estimated from the NOE cross peak buildup rates measured in 2D homonuclear NOESY spectra with different mixing times (50, 100, 150, 300 ms). The pyrimidine H5/H6 distance (2.41 Å) was used as the reference distance in these experiments. Other nonexchangeable proton distances were obtained from the 3D ^{13}C-edited NOESY-HSQC spectrum. NOEs between nonexchangeable protons were classified as strong, medium, weak, and very weak, with corresponding distance ranges from 1.8-3.0, 1.8-4.0, 1.8-5.0 and 1.8-6.0 Å, respectively. The systematic usage of 1.8 Å as the lower bound can be rationalized from errors in estimating interproton distances causing by internal motions and spin diffusion. Interproton distances between exchangeable protons, and exchangeable to nonexchangeable protons were derived from 2D NOESY, 3D ^{13}C/^{15}N-edited NOESY-HSQC and 3D ^{15}N-edited NOESY-HSQC spectra acquired in H_2O. Most NOE cross peaks involving exchangeable RNA protons were only classified as weak, except that the U NH to A H2 and G NH to C NH2 interactions in Watson-Crick base pairs were included as strong constraints. Because the existence of spin diffusion and

exchange phenomena could potentially affect the observable NOEs and complicate the interpretations of cross peak intensities, loose upper bounds of 4.0 or 5.0 Å were paced on base to sugar distances that can be clearly identified at short mixing times (100 ms). Canonical hydrogen bonds existing between known Watson-Crick base pairs and wobble G·U pairs were estimated from the significant downfield imino shifts and slow rates of exchange with solvent. Since there are few observable proton resonances within each base, hydrogen bonds were supplemented into the structure restraints. Two hydrogen bonds were used for each A-U pair; three for each G-C pair and two for each G·U wobble pair. Although the G56 H1 proton would satisfy at least one of the above criteria, hydrogen-bonding constraints were not included to avoid misidentification of acceptor atoms.

6.1.3.2. Dihedral angles

In RNA, the conformation of the sugar-phosphate backbone is defined by six torsion angles (α, β, γ, δ, ϵ and ζ), the ribose ring is defined by five endocyclic torsion angles (v_0, v_1, v_2, v_3, and v_4) and the orientation of the base relative to the sugar is given by the glycosidic angle χ (Saenger, 1984). Among the backbone dihedral angles, the only source of direct experimental information for α (P-O5') and ζ (O3'-P) are the ^{31}P chemical shifts; while measurable backbone angles β (O5'-C5'), γ (C5'-C4'), δ (C4'-C3') and ϵ (C3'-O3') involve either stereo-specific assignment of H5' and H5" and/or measurements of heteronuclear ^{1}H-^{31}P couplings. These backbone torsion angles were not obtained, either because the ^{31}P chemical shifts could not be assigned, or because of the severe overlapping of the proton resonances involved (H3', H4', H5', or H5"). Among the five torsion angles that define the ribose ring, only v_1 (C1'-C2') could be unambiguously obtained from the H1'-H2' coupling constants, since the chemical shifts of H1' protons have better dispersion and suffer less from chemical shift degeneracy and overlap. The H1'-H2' scalar coupling constants were measured in a 2D high-resolution DQF-COSY spectrum. Four nucleotides (G11, G56, A57 and G58) with H1'-H2' coupling constants larger than 8 Hz were classified as C2'-endo conformation. Five nucleotides (G5, G18, C19, A20 and A20) with observable H1'-H2' cross peaks and estimates of coupling constants between 2 and 8 Hz were classified as interconverting sugar puckers and thus were not included in the calculation. The

remaining nucleotides yielded no observable H1'-H2' cross peaks in either COSY or TOCSY spectra were classified as C3'-endo conformation. Except for G56, the χ glycosidic torsion angles (C1'-N1 or C1'-N9) for all other nucleotides were estimated to be in *anti* conformations. The established of the *syn* conformation for G56 is based on the strong H8 to H2' cross peak observed in short mixing time NOESY spectra.

Among the 12 total torsion angles in a nucleotide, only two torsion angles derived from experimental measurements (*i.e.*, sugar pucker v_1 and glycosidic χ) were used as dihedral restraints in the calculation. Typical uncertainties for the dihedral angle constraints were $\pm 30°$ for sugar puckers, and $\pm 120°$ for glycosidic torsion angles. The only nonexperimental constraints are the planarity restraints ($0° \pm 5°$) for those residues with amino groups (*i.e.*, G, C and A) which keeps these protons from flipping during the calculation. No other non-experimental constraints were added to introduce a bias towards idealized A-form RNA helices or perfect base-pair planarity.

6.1.4. Complex restraints

The distances between the L30 protein and RNA are derived from intermolecular NOEs observed from 2D and 3D experiments. Table 6.1 lists the observed NOEs between protein and RNA, which are converted into distance restraints for final structure calculation. Possible four hydrogen bonds between two protein side chains and two RNA bases are included only in the very last stage of the calculations.

6.2. Molecular Modeling

6.2.1. Theory

With the obtained experimental distance and dihedral restraints in hand, 3D structure calculations can be performed using the X-PLOR program (Brünger, 1992) incorporating the

Table 6.1: L30 protein-RNA NOEs. For simplicity, NOEs are group together by the order of protein residues (the same data as in Table 5.3 which was sorted by RNA order). For a more detailed list, see **Appendix C** for the entire NMR restraint set for molecular modeling

Protein	RNA	NOE intensity
Leu25 (H_N, α, β, γ, δ)	G56 (H1', 2', 3', H8)	very weak - medium
Gly26 (H_N, α)	G56 (H1', H8)	weak, medium
Ser26 (H_N)	G11 (H1)	very weak
Tyr27 (H_N)	G56 (H8)	very weak
Tyr27 (H_N, α, β, δ, ε)	A57 (H4', 5',")	very weak - medium
Tyr27 (H_N, α, β, δ, ε)	G58 (H1', 2', H8)	very weak - weak
Lys28 (H_N)	A57 (H4')	very weak
Lys28 (H_N, α, β, γ, δ, ε)	G58 (H1', 2', H8)	very weak - weak
Ser29 (H_N, β)	G11 (H1)	very weak
Ser29 (H_N, β)	G58 (H4')	very weak
Thr48 (H_N, α)	A57 (H2)	very weak
Pro49 (α, β, γ)	A57 (H2)	very weak - medium
Arg52 (γ, δ, ε)	A57 (H1', H8)	very weak - medium
Arg52 (γ, δ, ε)	G58 (H2', H8)	very weak - medium
Phe85 (β, δ, ε, ζ)	A55 (H2)	very weak - medium
Phe85 (β, δ, ε, ζ)	G56 (H2', 3', H1, H8)	very weak - medium
Val87 (H_N, α, β, γ)	G56 (H8)	very weak
Gly88 (H_N, α)	G56 (H8)	very weak

restrained molecular dynamics (rMD) method outlined in Figure 6.1.1. The protocol in rMD involves solving Newton's equation of motion subject to a simplified target energy function comprising terms for the experimental restraints (E_{exp}), covalent geometry ($E_{covalent}$), and non-bonded contacts ($E_{non-bonded}$), which can be expressed in following potential energy terms:

$$E_{total} = E_{covalent} + E_{non-bonded} + E_{exp} \tag{6.3}$$

where

$$E_{covalent} = E_{bond} + E_{angle} + E_{dihedral} + E_{improper} \tag{6.4}$$

$$E_{non-bonded} = E_{van\ der\ Waals} + E_{electrostatic} + E_{hydrogen\ bond} \tag{6.5}$$

and

$$E_{exp} = E_{NOE} + E_{cdih} \tag{6.6}$$

The covalent energy function includes bond (E_{bond}), angle (E_{angle}), dihedral ($E_{dihedral}$), and improper ($E_{improper}$, which is added to keep rings planar and to maintain the chirality of chiral centers) energy terms. The non-bonded energy function includes van der Waals ($E_{van\ der\ Waals}$), electrostatic ($E_{electrostatic}$) and hydrogen bond ($E_{hydrogen\ bond}$) terms. The initial velocities are assigned from a Maxwellian distribution corresponding to the desired temperature, commonly maintained using a temperature bath. Newton's equations of motion link the force acting on a particle to its acceleration. These can be solved by numerical integration over very small time steps to give both the velocity of the atom after the time step and the displacement of the atom during the time step. The time steps must be smaller than the time scales of the highest frequency motion in the system. Higher mass (*e.g.*, 100 amu) is assigned to hydrogen atoms in order to keep them with relatively low velocities. In determining structures from NMR data, experimentally determined distance and dihedral angle constraints need to be satisfied, which is achieved by adding more terms to the non-bonded potential. One means of applying the distance constraints is to use a square well potential of the forms:
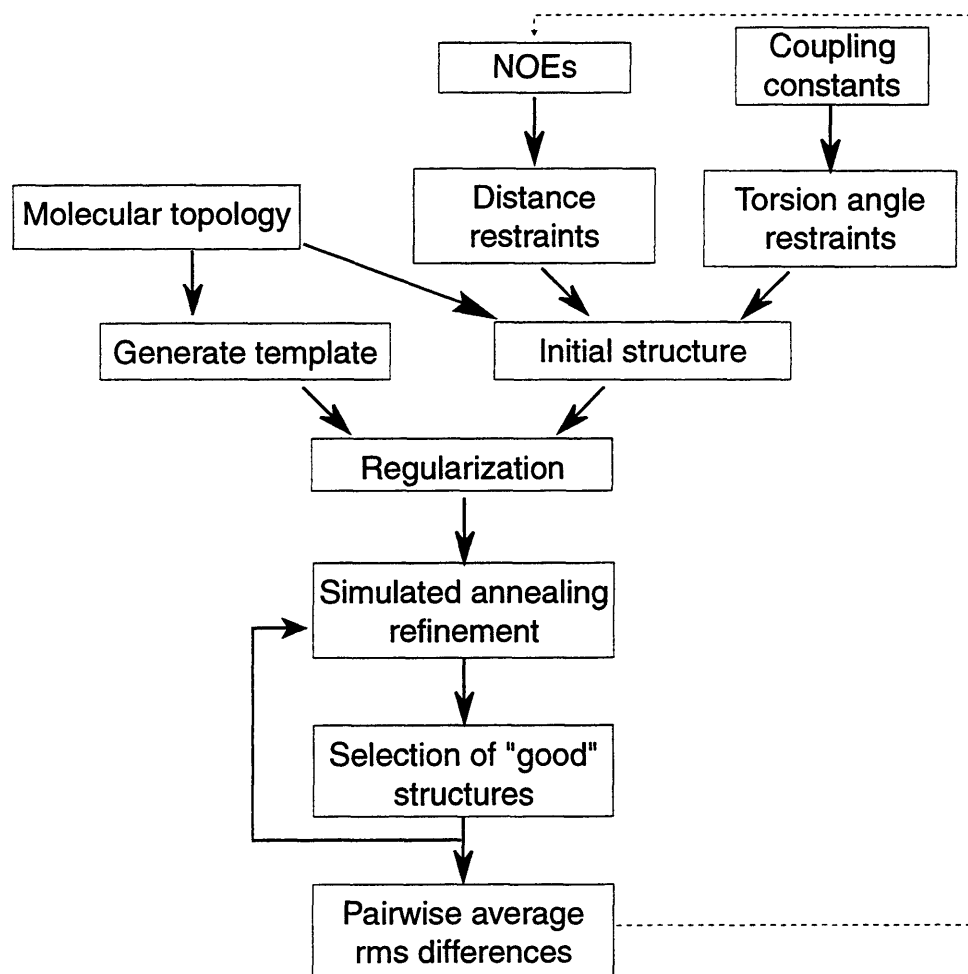
Figure 6.1.1: A representative scheme of NMR structure determination based on simulated annealing in X-PLOR (Brünger, 1992).

$$E_{NOE} = \begin{cases} k_{NOE} (d_{ij} - u_{ij})^2, \text{if } d_{ij} > u_{ij} \\ k_{NOE} (l_{ij} - d_{ij})^2, \text{if } d_{ij} < l_{ij} \\ 0 \text{ otherwise} \end{cases} \qquad (6.7)$$

Similarly, dihedral angle constraints can be incorporated using a square well potential of the form

$$E_{cdih} = \begin{cases} k_{tor} (\phi_{ij} - \phi''_{ij})^2, \text{if } \phi_{ij} > \phi''_{ij} \\ k_{tor} (\phi_{ij} - \phi''_{ij})^2, \text{if } \phi_{ij} < \phi''_{ij} \\ 0 \text{ otherwise} \end{cases} \qquad (6.8)$$

In order to generate structures carrying global minimal energy, a simulated annealing approach is used in the rMD. The underlying principle is to achieve global minimal energy by raising the temperature of the system and then slowly cooling it down. During the process, false local minima and large potential energy barriers along the path should be overcome, and consistency with the experimental restraints can be met by sampling the conformational space efficiently and comprehensively. The iterative cycle comes to an end when all of the experimental data have been interpreted without sacrificing covalent geometry and nonbonded contacts.

Based upon the above rMD theory, the structure calculation of the L30 protein-RNA complex structure was carried out with simulated annealing by generating initial structure from random coordinates and then further refining with the experimental restraints. Generally, there are two approaches to generate a complex structure, either through generating the complex as a whole, or through docking of the individually refined components. Directly generating the complex structure from random coordinates was not an ideal choice for the L30 complex. Since the total number of distance restraints used to generate the L30 protein-RNA complex structure exceeds 3,000, it requires large amounts of CPU time and prolongs each calculation step. In addition, the number of long-range distance restraints for the protein is significantly more than the RNA, and directly generating the complex structure gives very low structural convergence

(data not shown). The alternative, generating the individual components (namely the protein and the RNA) independently and then forming the complex through docking, gave much better results. More specifically, ensembles of the protein and the RNA structures were first generated independently using only intramolecular restraints. Then, the refined protein and RNA structures were docked under the influence of intermolecular distance restraints to give the complex structure; further refinement of the docked complex structure yielded the final structure of the complex.

6.2.2. Generating protein structures

6.2.2.1. Modeling protocols

The topology file for the L30 protein was prepared from X-PLOR using the parallhdg.pro parameter set using linear peptide coordinates generated from the Biopolymer Module in Insight II (MSI, Inc.). The 3D protein structures were generated on the basis of three protocols in the X-PLOR manual: (1) random.inp (Nilges *et al.*, 1988), (2) dgsa.inp (Nilges *et al.*, 1988) and (3) refine.inp (Nilges *et al.*, 1991). Slight modifications were made to adapt the calculation to the L30 protein, and the respective names were changed to randomhm.inp, dgsahm.inp and refinehm.inp (See Appendix B for details). The starting point for the structure generation was based on restraints with no stereospecific assignment and backbone torsion angle restraints. The first protocol (randomhm.inp) uses simulated annealing starting from a completely random array of atoms, with the choice of R^{-6} for distance averaging, and a soft-square potential as a restraining function for distance and torsion angle. The initial phase involved ramping up covalent geometry and distance restraints over total duration of 380 ps in dynamic calculations. There were 95 cycles of dynamic (100 integration time steps of 40 fs each) performed at 1000 K. While the force constants for van der Waals and improper energy terms were held constant at 0.1 kcal mol^{-1} Å^{-4}, and 0 kcal mol^{-1} rad^{-2}, respectively, bond, angle, and NOE terms were slowly increased at each cycle by a factor of 1.25. These constants were increased from 0.5 to 10.0 kcal mol^{-1} Å^{-2}, 0.25 to 5 kcal mol^{-1} rad^{-2}, and 0.0005 to 0.01 kcal mol^{-1} Å^{-2}, respectively. In the second

phase, three dynamic calculation (500 integration time steps of 3 fs each) were performed to increase the force constants for bond, angle, van der Waals terms from 20 to 100 kcal mol^{-1} Å^{-2}, 2.5 to 50 kcal mol^{-1} rad^{-2}, and 0.002 to 0.01 kcal mol^{-1} rad^{-4}, while holding the NOE force constants at 5 kcal mol^{-1} Å^{-2}. The second protocol (dgsahm.inp) was then used to regularize the initial structures by template fitting, which tests for the correct enantiomer and generates the missing atoms and then regularizes the coordinates. These structures were then refined through simulated annealing the third protocol (refinehm.inp). The structures were slowly cooled from 1000-100 K over a total duration of 10 ps. These were 18 cycles of cooling, each comprising 0.556 ps (111 integration time steps of 5 fs each), with a reduction in temperature of 20 K per cycle. During this period the force constants for bond, angle, improper, NOE and dihedral angle terms were held constant at 1000 kcal mol^{-1} Å^{-2}, 500 kcal mol^{-1} rad^{-2}, 500 kcal mol^{-1} rad^{-2}, 50 kcal mol^{-1} rad^{-2}, and 200 kcal mol^{-1} rad^{-2}, respectively, while the van der Waals term was increased from 0.003 to 4 kcal mol^{-1} rad^{-4} (the van der Waals radius scale factor decreases from 0.9 to 0.75). Finally, 200 cycles of Powell minimization were performed with the values of the various force constants set to their final values attained at the end of the cooling phase.

About 50% of the initial structures generated after these three steps had a significant small number of NOE and dihedral violations, and relatively lower total energy. Pairwise comparison of these structures indicates that these structures have a relatively low root-mean-square deviation (r.m.s.d.) for all heavy atoms (~3.0 Å), suggesting good convergence of the calculation. The other 50% of structures not only had higher total energy, in particular a much higher number of NOE violations, but also showed a significantly large r.m.s.d. compared to the converged structures. The nonconverging structures were discard without further analysis.

6.2.2.2. Iterative refinements

Next, the initial generated structures were subjected to iterative refinement using the "refinehm.inp" protocol. Despite the first three steps that generated structures with relatively good initial convergence, the initial structures carried a large number of NOE violations (over 100). This can be examined from the distribution of violations in the ensemble of calculated

structures. The apparent violations could be resulted from three sources. First, systematic NOE violation might occur because of possible incorrect assignments and the existing of ambiguous assignments from degeneracy in the NOESY experiments. The accuracy of the assignments with interproton distances greater than 6 Å was double-checked with the NOESY spectra. Since the molecular modeling requires that restraints involving ambiguous NOEs must be consistent with the current structure, unreasonable assignments with interproton distances greater than 6 Å were eliminated. Second, since the 3D heteronuclear-edited NOESY experiments were recorded with mixing times of 100 to 150 ms, it was apparent that the classification of NOEs into strong, medium, weak and very weak categories could be influenced by spin-diffusion effects. Consequently, NOEs in the restraint list were evaluated for spin-diffusion effects on the basis of observed distances and spatial relationships in the convergent ensemble of structures. If the resulting distance was significantly longer than that predicted from the observed NOE intensity, and a spin diffusion pathway was highly probable on the basis of the spatial geometry of the relevant protons, the corresponding upper bound distance limit for the NOE was either adjusted upward to the next class, or removed from the restraint list if the NOE was originally classified as very weak. The procedure was employed specifically for three types of situations: (1) where two methylene (or methyl) protons exhibit an NOE to the same spin, but one of the two protons clearly points away from this spin at a distance greater than 6 Å; (2) where the complete ring system of a Phe (or Tyr) shows NOEs to a particular spin, but it is clear from the spatial configuration that only one aromatic proton is close in space to this spin; (3) as the approximate interproton distance restraints are classified into four ranges, the resulting distinct cutoffs can result in systematic errors for interproton distances whose values lie at the boundary of two distance ranges. Thus, if a particular interproton distance restraint was systematically violated in all of the structures, it was reclassified into the next class.

With each round of iterative refinement, more experimental restraints were corrected as the quality of the structure improved. More importantly, the current ensemble of refined structures also aided in the identification of misidentified NOEs that arose as a result of the very limited ^{1}H chemical shift dispersion, therefore more restraints were added into the calculation.

Simulated annealing calculations were then repeated after each update of the restraint list, and a new set of structures was generated for further analysis with NMR data.

6.2.2.3. Stereospecific assignments

The refined structures also assisted in the stereospecific assignments, in particular, many methyl groups with prochiral centers. At the end of the iterative refinement cycle, stereospecific assignments were obtained for 41 residues with β-methylene protons, and for 12 Leu and 8 Val residues with prochiral $(CH_3)_2C$ groups. Residues without stereospecific assignments correspond to either surface residues with disordered side chains, or residues with degenerate β-methylene protons, or residues with chemical shifts masked by overlaps in NMR spectra. The validity of stereospecific assignments was subsequently confirmed by being properly accounted for in further refined structures.

For well-defined Phe and Tyr residues, the NOE distance restraints to the H_δ and H_ϵ protons can often be assigned to only one side of the ring, thereby discriminating between the $H_{\delta 1}$ and $H_{\delta 2}$ or the $H_{\epsilon 1}$ and $H_{\epsilon 2}$ protons (Powers *et al.*, 1993). In the L30 protein, all six Tyr and two Phe residues have well defined conformations, making it possible to assign NOE restraints to only one of the pair of H_δ and H_ϵ protons when it was clear that the NOE could only be from one face.

The incorporation of the torsion angle restraints mainly assists in the convergence rate of the calculation, particularly since the torsion angle restraint boundaries were always much larger than the resulting standard deviation for the corresponding torsion angles in the calculated ensemble of structures.

After properly correcting all mischaracterized distance restraints and implementing the stereospecific assignment of many prochiral centers and aromatic residues, the simulated annealing in restraint molecular dynamic calculation procedure was repeated once more. In the end, starting with generating initial structures from random coordinates and repeating with three

rounds of iterative refinement, about 90% of the calculated structures converged with no significant violation energies that is larger than 400 kcal mol^{-1}.

6.2.3. Generating RNA structures

6.2.3.1. Modeling protocols

Unlike the protein structures, the RNA structures were generated from a slightly different approach involving modifying the "random.inp" and "refine_gentle.inp" protocols (see Appendix B for details) in the X-PLOR manual. Instead of starting from random coordinates, an A-form RNA with double stranded upper and lower stems was used to start the calculation. R^{-6} was chosen for distance averaging, and a soft-square potential for the restraining function. The first phase was the "rnainit.inp" protocol involving ramping up covalent geometry and distance restraints over a total duration of 63 ps in dynamic calculation. There were 21 cycles of dynamic (1000 integration time steps of 3 fs each) performed at 1000 K. While the force constants for van der Waals and improper energy terms were held constant at 0.1 kcal mol^{-1} Å^{-4} and 0 kcal mol^{-1} rad^{-2}, respectively, bond, angle, and NOE terms were slowly increased in each cycle by a factor of 1.25. These constants were increased from 0.5 to 10.0 kcal mol^{-1} Å^{-2}, 0.25 to 5 kcal mol^{-1} rad^{-2}, and 0.5 to 50 kcal mol^{-1} Å^{-2}, respectively. In the second phase, the force constant for the van der Waals term was ramped up from 0.01 kcal mol^{-1} rad^{-4} to its full value (1 kcal mol^{-1} rad^{-4}) over 36 ps dynamic while holding the bond, angle and NOE force constants at their current value. There were 12 cycles of dynamics with 1000 integration time steps of 3 fs each. In the next step using the "rnarefine.inp" protocol, the structures were refined through molecular dynamic calculation and conjugate gradient minimization. Throughout the refinement, the force constants for bond, angle, improper, NOEs, dihedral angles and van der Waals were held constant at 1000 kcal mol^{-1} Å^{-2}, 500 kcal mol^{-1} rad^{-2}, 500 kcal mol^{-1} rad^{-2}, 50 kcal mol^{-1} Å^{-2}, 200 kcal mol^{-1} rad^{-2}, and 1 kcal mol^{-1} rad^{-4}, respectively. Two hundred cycles of Powell minimization were performed preceding the three dynamics cycles of 10 ps (10000 integration time steps of 1 fs each) at 1000 K, 600 K

and 400 K. Finally, 200 cycles of Powell minimization were performed with the values of the various force constants set to their final values attained at the end of the cooling phase.

6.2.3.2. Iterative refinements

Similar to the protein structure calculations, iterative refinements were also performed in order to correct errors associated with misassignment of NOEs and incorrect torsion angle restraints. In the end, structures with no NOE violations larger than 0.3 Å were subjected to pairwise comparison. The convergence in the RNA structures was significantly smaller (only about 50%) than that of the protein, even though many of the RNA structures have no apparent NOE violation, but show a significantly larger r.m.s.d. (> 2.5 Å for all heavy atoms). The main reason for the apparent discrepancy relies on the lack of long-range NOEs to define the orientation of the two helical stems separated by the internal loop. Therefore, the selection of the converged structures was based on the best superimposed internal loops rather than the overall r.m.s.d. of the whole RNA ((< 1.8 Å for all heavy atoms).

6.2.4. Docking of complex

6.2.4.1. Docking protocols

After the structures of both protein and RNA had been calculated and individually refined under intramolecular distance constraints, they were docked by simulated annealing. The coordinates of the protein were first rotated up to 360° and placed 100 Å away from those of the RNA in a random orientation. The protein and the RNA were then brought together slowly through the intermolecular NOEs. Briefly, while holding the intramolecular NOE force constant at 50 kcal mol^{-1} A^{-2}, the intermolecular NOE force constant was slowly ramped up from 0.001 to 50 kcal mol^{-1} A^{-2} over a total duration of 156 ps. There were 26 cycles of NOE ramping, each comprising 6 ps (2000 integration time steps of 3 fs each), with the NOE force constant increased at each cycle by a factor of 1.5. In all cases, convergence to the same ensemble of structures was achieved (~85%). The complex structures were further refined through constant temperature

simulated annealing and slow cooling to obtain the final structures using the refinement procedure described for the RNA calculations. The target function that was minimized during simulated annealing comprises only quadratic potentials for the covalent geometry, a square-well quadratic potential for the experimental distance and torsion angle restraints together with a quartic van der Waals repulsion term. No classical empirical energy terms for electrostatic or hydrogen-bonding interactions were present in the target function, which prevents the false contacts influenced by these interactions.

Initially, it was thought that there might be a problem associated with using only a repulsion term for nonbonded contacts. In general, if the nonbonded contacts are only represented by a simple quartic van der Waals repulsion term, the only attractive forces present in the target function are those associated with the interproton distance restraints. Hence, the lower the number of interproton distance restraints, the smaller the contribution of the attractive forces to the target function, which will tend to result in a systematic expansion of the calculated structures. This was primarily a concern for the RNA structures that were calculated with less long-range distance restraints. An alternative that includes Leonard-Jones potential containing both attractive and repulsion terms was used in the refinement; no apparent r.m.s.d. changes between the RNA structures generated using Leonard-Jones potential function and those generated from a repulsion function were observed.

6.2.4.2. Hydrogen bonds

In the very final stage of the calculations, intermolecular hydrogen bonding restraints between the protein side chains of Asn47 and Arg52 to RNA bases were tested. The side chains of these residues are buried at the protein-RNA interface and interact with the RNA. Since no direct intermolecular NOEs involving guanidino protons of Arg52 or the amide side chain of Asn48 were observed, the exact location of the donor and acceptor groups could not be determined from NMR. Evidence that the functional groups of the interacting arginines are either buried at the interface or involved in some sort of electrostatic interaction comes from the observation of the Hϵ and guanidino protons in the complex. Hϵ-Nϵ cross peaks for all the Args

were observed in the ^{1}H-^{15}N correlation spectrum of the complex, with ^{1}H and ^{15}N shifts in the range of 6.8-7.2 and 82-83 ppm, respectively. Three patches of four broad cross peaks arising from the hydrogen bonded guanidino groups of Args were also observed in the region from 6.5-7.9 ppm in the ^{1}H dimension and 61-73 ppm in the ^{15}N dimension. In addition, weak NOEs between the Hε and guanidino protons were observed in the 3D ^{15}N-separated NOE spectrum. In contrast to the bound form, in the free protein, Arg52 has a single Nε-Hε cross peak (with ^{1}H and ^{15}N chemical shifts of 7.43 and 84.6 ppm, respectively, similar to those of a free Arg) and no guanidino cross peaks were observed in the ^{1}H-^{15}N correlation spectrum. Line broadening of the guanidino cross peaks is mainly due to rotation about the Nε-Cζ partial double bond, as well as flipping about the Cζ-Nη bonds (Henry & Sykes, 1995). A similar chemical exchange mechanism involving rotation about a C-N bond is responsible for broadening of the proton resonances of the 6-NH$_2$ and 2-NH$_2$ groups of adenine and guanine, respectively, both of which are involved in Watson-Crick base pairing. Similar to the Arg52 guanidino group, the N$_{82}$-H$_{82}$' and N$_{82}$-H$_{82}$" cross peaks for Asn48 are also broad, impeding the observation of any intermolecular NOEs from H$_{82}$' and H$_{82}$" protons of Asn48 in the ^{15}N-separated NOE spectrum. Line broadening of Asn side-chain amides involved in hydrogen bonding with RNA bases has been previously observed in the Rev-RRE complex (Battiste *et al.*, 1996).

The calculation strategy employed for the interfacial protein-base hydrogen bonding restraints involved two types of distance restraints: (i) 'repulsive' distance restraints with a lower bound of 4 Å (and an unrestrained upperbound); and (ii) ambiguous distance restraints (with a range of 2.4 -3.3 Å), represented by the $(\Sigma r^{-6})^{-1/6}$ sum, between donor groups of these side chains and all possible acceptor groups of the RNA bases. It was known that the introduction of the interfacial hydrogen bonding restraints had no effect on the overall structure of the complex, but this improved local convergence and precision for the Nε and guanidino groups of Arg52 and the carboxyamide of Asn48. Thus, excluding the latter atoms, the precision of the coordinates (protein backbone and side chains and the RNA) remains unchanged. The r.m.s.d. of the mean coordinate positions obtained with and without interfacial hydrogen bonding restraints is only 0.2 Å, which is well within the errors of the coordinates.

6.3. Structure Statistics

6.3.1. Overall statistics

The final ensemble of structures of the L30 protein-RNA complex was determined on the basis of 3358 NMR experimental restraints summarized in Table 6.2 (see Appendix C for details), in conjunction with the inter-molecular hydrogen bonding restraints discussed above. These comprise distance restraints of 2241 protein and 804 RNA intramolecular, 90 intermolecular (protein-RNA), and torsion angle restraints of 127 in protein and 106 in RNA. The protein interproton distance restraints are subdivided as follows: 902 intraresidue, 538 interresidue sequential ($|i\text{-}j| = 1$), 380 interresidue short-range ($1 < |i\text{-}j| \leq 4$), 363 interresidue long-range ($|i\text{-}j| > 4$) NOE derived restraints, 58 backbone hydrogen bonds. The RNA distance restraints include 428 intraresidue, 224 interresidue sequential ($|i\text{-}j| = 1$), 78 interresidue long-range ($|i\text{-}j| > 1$) NOE derived restraints and 74 base pair hydrogen bonds. The torsion angles comprise 75 backbone ϕ and 52 χ_1 for protein and 33 glycosidic, 44 sugar pucker and 29 amino group in the RNA.

The distance restraint distribution of the protein and RNA are further summarized the charts shown in Figure 6.3.1, with panel **a** for the protein and panel **b** for the RNA. As can be seen, the average mount of intra-molecular distance restraints is 22 per residue in the protein (providing that each restraint is only counted once for paired protons), and 24 per residue in the RNA. Although the average intra-molecular distance restraints per residue are similar, the amount of long range distance restraints ($|i\text{-}j| > 4$ for protein and ($|i\text{-}j| > 1$ for RNA) that help to define the tertiary structure is significant more in the protein than in the RNA.

A summary of structural statistics based on the ensemble of 31 final simulated annealing structures of the complex is given in Table 6.3. As is evident from the data, there are no interproton distances and torsion angle violations greater than 0.5 Å and 3°, respectively which suggests good convergence and precision of the calculated structures. Two views of a

Table 6.2: Summary of constraints for L30 protein-RNA complex structure calculations.

Types of restraints	Sum of restraints	Average restraints per residue
Total restraints	**3358**	**24.5**
Total distance restraints used for the complex	3125	22.8
Protein distance restraints	2241	21.5
intraresidue NOEs	902	8.7
interresidue NOEs	1281	12.3
sequential NOEs ($|i\text{-}j| = 1$)	538	5.2
strong	37	0.4
medium	180	1.7
weak	164	1.6
very weak ·	157	1.5
short range NOEs ($1 < |i\text{-}j| \leq 4$)	380	3.7
strong	5	0.0
medium	86	0.8
weak	119	1.1
very weak	170	1.6
long range NOEs ($|i\text{-}j| > 4$)	363	3.5
strong	10	0.1
medium	85	0.8
weak	142	1.4
very weak	126	1.2
hydrogen bond restraints	58	0.6
RNA distance restraints	804	24.4
intraresidue NOEs	428	13.0
interresidue NOEs	376	11.4
sequential NOEs ($|i\text{-}j| = 1$)	224	6.8
strong	19	0.6
medium	52	1.6
weak	98	3.0
very weak	55	1.7
long-range NOEs ($|i\text{-}j| > 1$)	78	2.4
strong	9	0.3
medium	26	0.8
weak	34	1.0
very weak	9	0.3
hydrogen bond restraints	74	2.2
Protein-RNA inter-molecule NOE restraints	80	0.6
strong	0	0.0
medium	11	0.1
weak	28	0.2
very weak	41	0.3
Dihedral angle constraints	233	1.7
Protein backbone ϕ	75	0.7
Protein backbone χ_1	52	0.5
RNA glycosidic χ	33	1.0
RNA sugar pucker δ and v_2	44	1.3
RNA amino groups	29	0.9
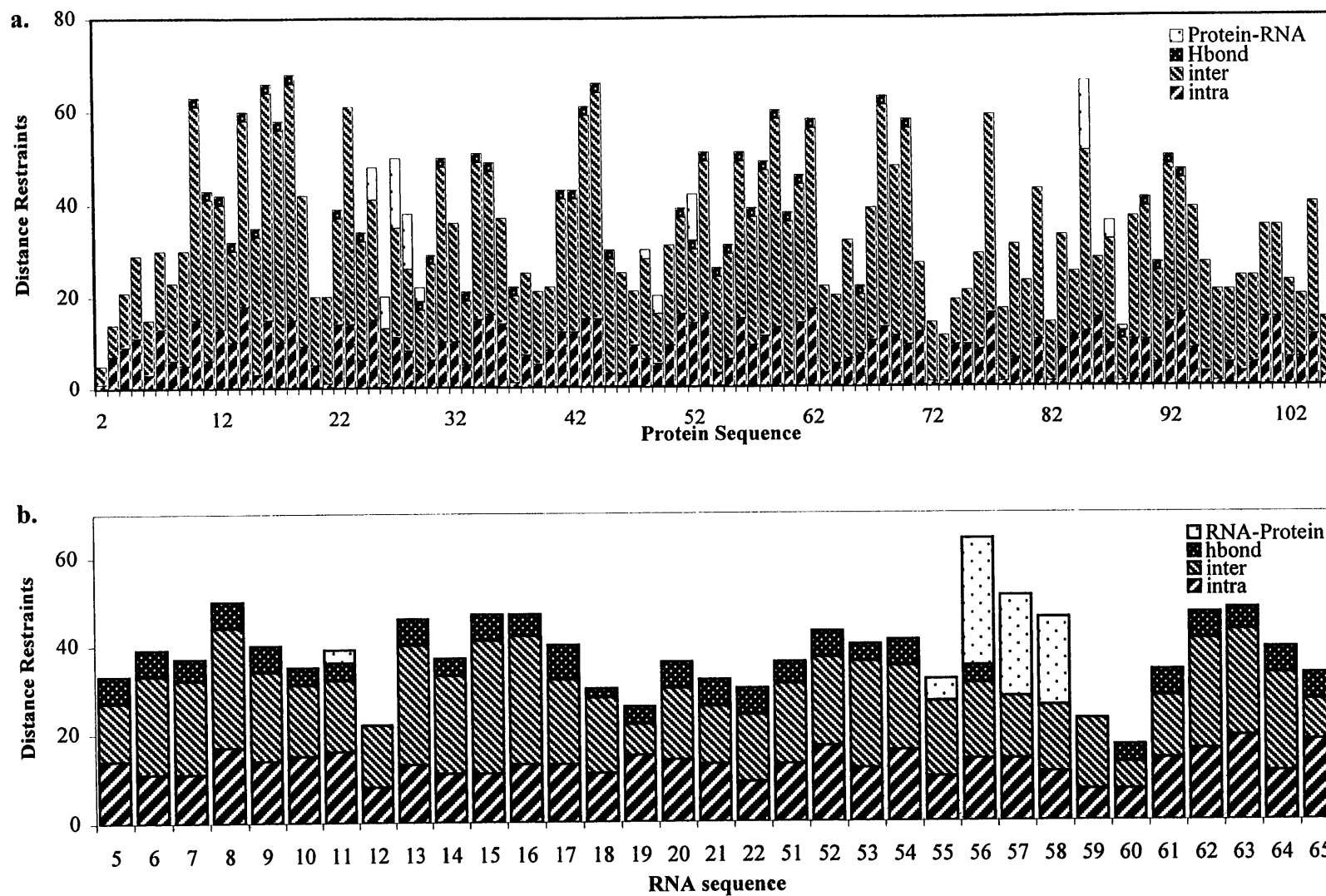
Figure 6.3.1: The number of NOE distance restraints for each residue in **a**, the L30 protein and **b**, the RNA. Intraresidue restraints are counted only once; interresidue and intermolecular restraints are counted twice, once for each residue involved.

Table 6.3: Summary of structural statistics of the L30 protein-RNA complex based on simulated annealing (SA) calculations using the X-PLOR program.

	{SA}	<SA>r
R.m.s.d from NOE distance restraints $(\text{Å})^2$	0.026 ± 0.003	0.027
R.m.s.d from expt. dihedral angle restraints (deg)	0.684 ± 0.086	0
R.m.s.d from idealized covalent geometry		
Bond (Å)	0.003 ± 0.0002	0.003
angles (deg)	0.534 ± 0.012	0.538
Impropers (deg)	0.631 ± 0.051	0.646
Restraint violations		
number of distance violations > 0.2 Å	10.9 ± 4.1	10
largest distance violation (Å)	0.4	0.29
number of dihedral violation > 2°	1.3	2
largest dihedral violation (deg)	3.1	4.9
Final energies (kcal mol^{-1})	555.2	604.9
bond	27.5	29.2
angle	222.8	224.8
improper	46.0	48.0
van der Waals	152.2	190.1
distance restraints	106.5	112.6
dihedral angles	0.2	0

The notation of the NMR structures is as follows: {SA} refers to the final ensemble of 31 accepted structures. <SA> refers to the average structure obtained by averaging the coordinates of the individual SA structures best fitted to each other (with respect to all the heavy atoms). <SA>r refers to the restrained minimized average structure. For the ensemble, the average value plus or minus the standard deviation is quoted.

superposition of the 31 final simulated annealing structures of the complex are shown in Figure 6.3.2 and Figure 6.3.3. The overall non-hydrogen atom pairwise rmsd is about 2.65 ± 0.70 Å. This relatively high r.m.s.d. came from two separated regions. Residue 2-8 and 101-105 at the N and C termini, respectively, appear to be disordered in solution since only intraresidue and sequential interresidue NOEs are observed. The intrinsic flexible conformations of these two segments in the protein were independently corroborated by the ^{15}N backbone dynamic studies, in which the relaxation parameters suggests rapid motions at these two segments (see Chapter 4.2.6). The other source of the high overall r.m.s.d. of the complex structures comes from the orientations of the two RNA helices; this is expected because of the occurrence of RNA bending at the internal loop, and no long-distance restraints could be obtained in the NMR observable. Taking these factors into the account, excluding the two RNA helical region and N- and C-termini of the protein, the precision is 1.39 ± 0.10 Å for all heavy atoms in the core of the complex (residues 9 to 100 in the protein plus residues 11-12, 55-59 in the RNA).

6.3.2. Protein statistics

Within the L30 complex, the precision of the protein is much better than the RNA because an overwhelming larger amount of long-distance restraints were obtained from the NMR data to define the protein structure. As can been seen in Figure 6.3.4, the ensemble of protein backbones in the complex form are significantly organized on the basis of best fitting to protein backbones. The precision of the protein coordinates is 1.07 ± 0.30 Å for all the protein backbone (N, Ca, C, and O atoms) in the complex using pairwise rmsd analysis. Excluding the poorly defined residues 2 to 8 and 101 to 105 at the respective N- and C-termini, the precision of the protein coordinates is 0.60 ± 0.09 Å for the backbone atoms (N, Ca, C, O atoms). The backbone of the core of the protein comprising the elements of regular secondary structure is defined to an even greater degree of precision and accuracy. Among the regular protein secondary elements (*i.e.*, α-helices and β-strands), residues 9 to 18 show the best precision, 0.12 ± 0.03 Å, and residues 75 to 81 show the least precision, 0.26 ± 0.11 Å. The differences in the structural precisions are directly related to the amount of distance restraints used in the structure calculation

(shown in Figure 6.3.1). Not surprisingly, surface side chains, and in particular residues with longer side chains such as lysine and arginine, tend to be partially disordered, especially beyond Cγ position.

6.3.3. RNA statistics

In contrast to the protein, the precision of the RNA is significantly less, with an overall 2.23 ± 0.53 Å calculated on best superposition of all the RNA heavy atoms (C, O, N and P). As mentioned earlier, this less than ideal precision is directly resulted from the orientation of the two helices, and the ends fraying at the RNA termini. The respective local structures of the two helices are quite good, with 1.37 ± 0.34 Å for the lower stem (residues 5-10 and 60-65), and 0.98 ± 0.22 Å for the upper stems (residues 13 to 17 and 50 to 54). By only taking into account of the RNA internal loop (residues 11-12 and 55 to 59), the precision is increased significantly, with 1.17 ± 0.27 Å. In particular, an increase in the number of restraints not only reduces the atomic rms spread of the ensemble of simulated annealing structures but also results in a concomitant and significant increase in the accuracy of both the ensemble and means coordinates.

In summary, the distance information derived from NOE measurement and the torsion angle information obtained from the three bond *J*-coupling measurement allowed the molecular modeling to be carried out for the NMR derived the L30 protein-RNA complex structure. 3358 of experimentally derived restraints, including 3125 distance restraints and 233 torsion angle restraints are input into the complex modeling that was out carried out on the basis of simulated annealing in restraint molecular dynamic in the X-PLOR program. The strategy for generating the complex structure involved generating the individual protein and RNA component independently with intra-molecular restraints, and then docking the protein and RNA together under the influence of inter-molecular distance restraints. An iterative refinement approach was used to identify the unassigned, or misarranged restraints. The improved experimental restraints in return facilitate the convergence of the final calculated structures. The final ensemble of
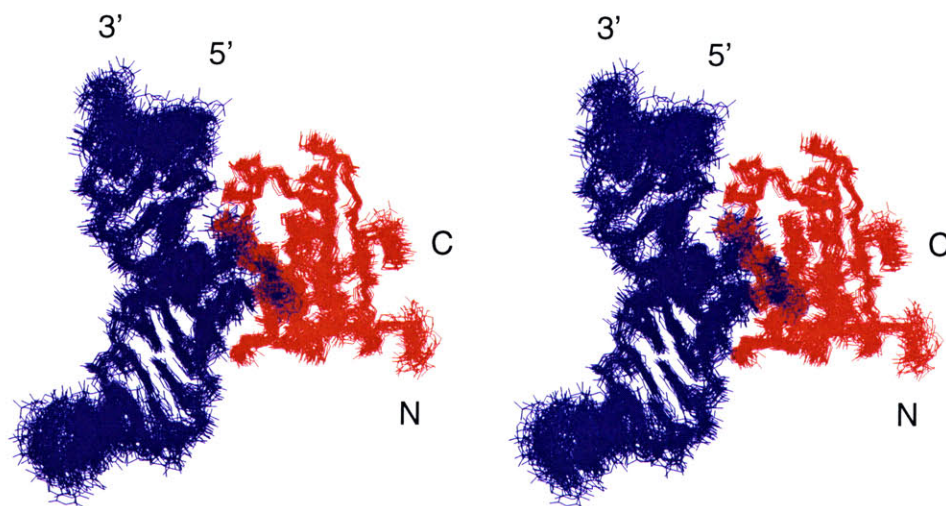
Figure 6.3.2: Stereo view of a best fit superposition of 31 simulated annealing structures of the L30 protein-RNA complex. This view shows the major groove of the internal loop of the RNA. The N- and C-termini of the protein are labeled as well as the 5' and 3' ends of the RNA. Only the non-hydrogen backbone atoms are shown for the protein, and no hydrogens are represented in the RNA.
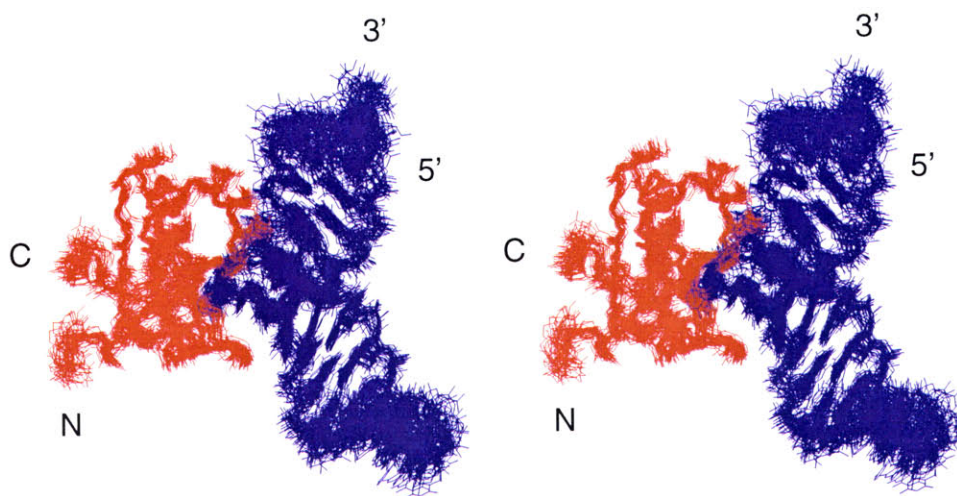
Figure 6.3.3: Stereo view of a best fit superposition of 31 simulated annealing structures of the L30 protein-RNA complex. This view shows the minor groove (as compared to the major groove in Figure 6.3.2) of the internal loop of the RNA. The N- and C-termini of the protein are labeled as well as the 5' and 3' ends of the RNA. Only the non-hydrogen backbone atoms are shown for the protein, and no hydrogens are represented in the RNA.
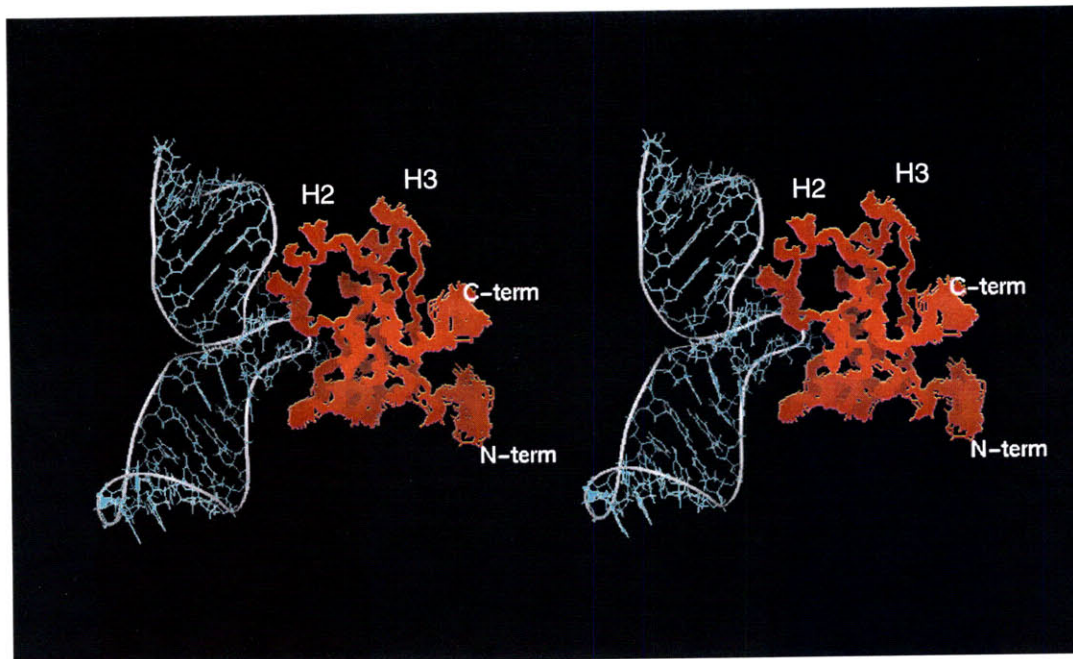
Figure 6.3.4: Stereo view of a best fit superposition of 31 simulated annealing structures of the L30 protein in the complex. The N- and C-termini of the protein are labeled as well as Helix 2 and Helix 3. Only the non-hydrogen backbone atoms are shown for the protein.

structures consists of 31 accepted SA structures, which were selected on the basis of minimal total energy. The structural core of the complex, which consists of approximately 90 ordered protein residues and 10 residues within and flanking the internal loop of the RNA, has a root-mean squared (r.m.s.) difference of 1.39 ± 0.10 Å for all heavy atoms of the core residues in complex between the 31 superposition structures. When the entire molecule is considered, including the disordered the N-and the C-termini of the protein, and the less defined orientations of the two RNA helices gave rise to the major structural r.m.s deviation (2.65 ± 0.70 Å) for all heavy atoms. These regions are locally order, but globally disordered because of the lack of long range restraints.

CHAPTER 7. Structure of the L30 Complex

7.1. Structure Description of the L30 Complex

The unambiguous identification of various intra- and inter-molecular NOEs and accurate measurement of many coupling constants yielded an unusually complete set of experimental restraints for structural modeling in X-PLOR, leading to an ensemble of well-defined L30 protein-RNA complex structures with high-resolution. The overall view of the complex from the superimposed 31 structure presented in Figure 6.3.2 and 6.3.3 shows the intimate contacts between the protein and RNA. Detailed analyses of this ensemble of structures and the average structure of the complex allowed the identification of the major structural elements present in both the protein and the RNA. Careful examination of the interfaces between the protein and RNA in the complex should facilitate the identification of structural and functional elements that are important for the protein-RNA recognition. The protein and the RNA structures are described in detail below.

7.1.1. Protein structure

7.1.1.1. Secondary structural elements observed in NMR analysis

The patterns of sequential and short-range NOE cross peaks, the $^3J_{HNH\alpha}$ coupling constants, and the slowly exchanging amide protons for the L30 protein, which all give indications of the secondary structure of the protein, were summarized in Figure 4.2.8 on page 92. Four regions of α-helical structure are clear from the patterns of short range NOEs (i to $i+3$, i to $i+4$) and are further supported by relatively strong sequential amide-proton to amide-proton cross peaks and small $^3J_{HNH\alpha}$ coupling constants. Four regions of β-strand are also obvious from the pattern of strong sequential H_N-H_α (i to i-1), weak or absent sequential amide to amide proton cross peaks and large $^3J_{HNH\alpha}$ coupling constants. The hydrogen-bonding patterns were also

obvious for all helices and strands, judging from the slowly exchanging amide resonances. In the helical regions, the D_2O exchange rates for amide protons of the first three to four residues are much faster, comparing to those involving hydrogen bonds within helices. In the β-sheet region, D_2O exchange rates of the amide protons at the edge of Strand A and strand C showed an alternate pattern: those exposed to the solvent have a much faster solvent exchange rate than the alternate amides involving hydrogen bonds with the inner strands. On the contrary, the amide protons of Strand B and Strand D have homogeneous slow D_2O exchange rates, resulting from the formation of hydrogen bonds at both sides of each strand.

7.1.1.2. Overall description of the protein tertiary structure from molecular modeling

The experimentally observed secondary structure elements in the L30 protein can be clearly seen in the final ensemble of tertiary structures shown in stereoview in Figure 7.1.1. The L30 protein 3D structure can be best characterized as an α–β sandwich, in which the four-stranded β-sheet is sandwiched between two layers of α-helices. The four strands, indicated in alphabetical order from N- to C-terminal, comprise the following residue numbers: Strand A (Lys22 - Gly26); Strand B (Leu41 - Ala45); Strand C (Thr65 - Phe70) and Strand D (Val89 - Leu93). The four helices, indicated in numerical order, comprise the following residue numbers: Helix 1: Glu8 - Ile18; Helix 2: Thr30 - Gly37; Helix 4: Leu51 - Leu62; and Helix 4: Glu76 - Gly82. A schematic representation is shown in Figure 7.1.2. A unique characteristic of the protein structure is the arrangement of the four β-strands. Starting with Strand A at one edge of the β-sheet, three strands (*i.e.*, Strands A, D and B) run anti-parallel to each other. But Strand C, at the far edge of the β-sheet in Figure 7.1.2, reverses the antiparallel order by running parallel to Strand B. A network of hydrogen bonds connects the backbones of the adjacent β-strands into a β-sheet platform, allowing two sets of parallel α-helices to pack on each side of the β-sheet; all β-strands are bridged through α-helices. In this arrangement of secondary structures, Helix 2 and Helix 3 run almost parallel to each other and are packed on one side of the β-sheet platform, while Helix 1 and Helix 3 are on the other side of the β-sheet. In addition to these regular structures (namely α-helices and β-strands), there are six loops connecting these elements. The first loop, adjoining Helix 1 and Strand A, is established by a four-residue turn (residues 19-22).

The second loop (27-29) turns Strand A into Helix 2. Loop 3 (37-40) joints Helix 2 and Strand B. The forth loop (45-50) connects Strand B and Helix 3, which is connected to Strand C by a tight-turn (63-65). The fifth loop (71-75) bridges Strand C and Helix 4, and the sixth loop (83-88) connects Helix 4 and Strand D. The backbone is completed with a coil (94-105) at C-terminus.

7.1.1.3. Hydrophobic core of the protein

As can be seen in the ensemble of the superimposed structures, the protein is stabilized by various interactions. Besides a network of hydrogen bonds that connects the backbones of each helix and the adjacent β-strands, extensive hydrophobic residues play critical roles in stabilizing the protein core. First, the most direct observations lie within the β-sheet, the core structural element of the protein. The slight right-handedness of the curved β-sheet provides a hydrophobic platform for the packing of the α-helices on both sides. There are a number of phylogenetically conserved hydrophobic and aromatic residues on both sides of the β-sheet. These residues not only stabilize interactions between strands, but also act as contacting points for orienting the helices on both sides of the β-sheet (Figure 7.1.3). More specifically, in the upper layer of the protein (facing towards Helix 2 and Helix 3), hydrophobic contacts are seen at residues Val67 and Tyr69 of Strand C, Ile42 and Ile44 of Strand B, and Val 89 of Strand D. These hydrophobic and aromatic residues on the top layer of the β-sheet make further contacts to residues Val31 and Leu34 from Helix 2, and Leu56 and Tyr59 from Helix 3. These interactions not only help to define the orientations of Helix 2 and Helix 3 with respect to the β-sheet, they also define the orientations between these two helices. Furthermore, there are a few clusters of hydrophobic residues on the bottom layer of the β-sheet (facing towards Helix 1 and Helix 4). These residues, namely Tyr68 and Phe70 of Strand C, Ile43 of Strand B, and Val90 and Ile92 from Strand D, form the lower core of the protein with residues Ile10, Leu14 and Ile18 on Helix 1, and Leu77 and Val81 on Helix 4. Among them, Ile43 from Strand B is the key hydrophobic residue in holding three of the β-strands (B, C and D) and two of the α-helices (1 and 4) close together. The critical role of Ile43 is supported by an *in vivo* experiment showing a slow growth
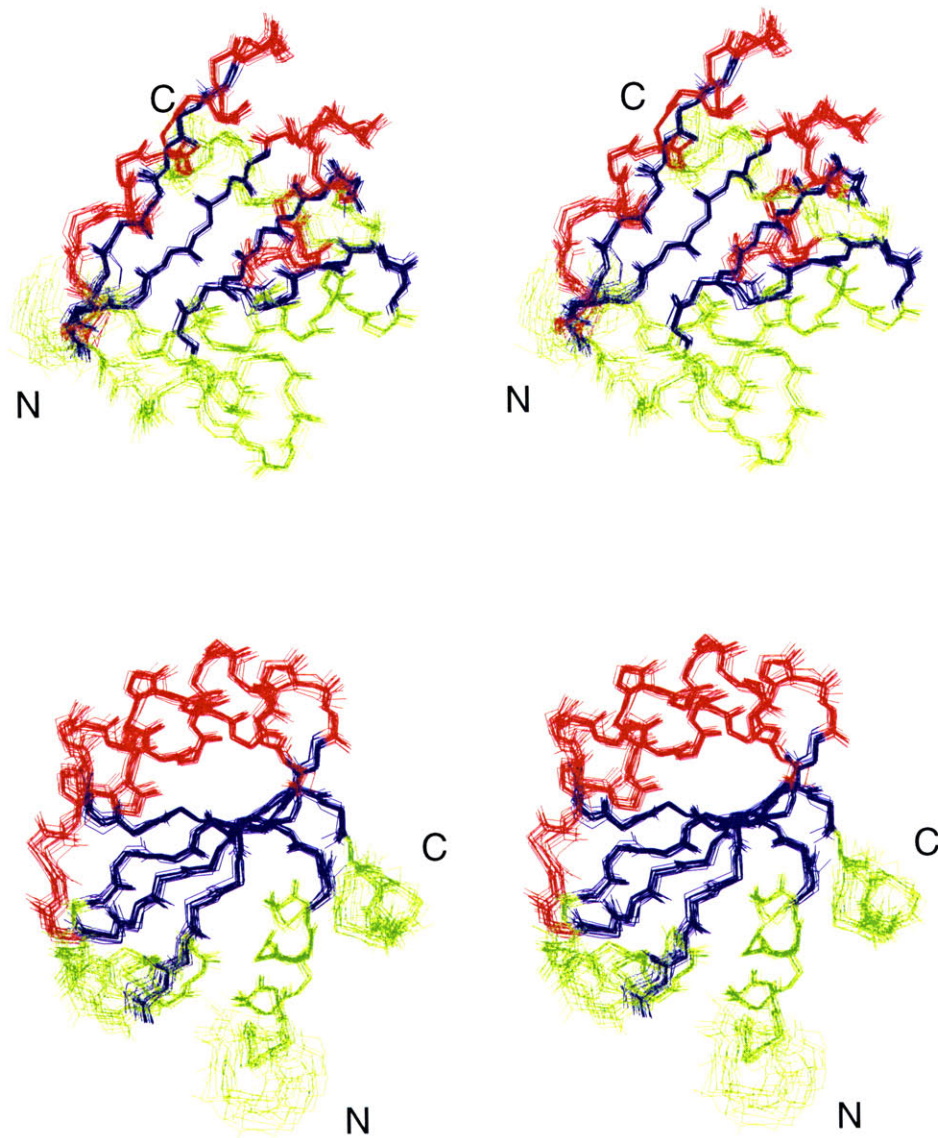
Figure 7.1.1: Two stereo views of a best fit superposition of 19 simulated annealing structures of the L30 protein in the bound form. The N- and C-termini of the protein are labeled, and only the non-hydrogen backbone atoms are shown. In green are the N-terminus (Helix 1), Helix 4 and the C-terminus. The four-stranded β-sheet is shown in blue, and Helix 2 and Helix 3 are depicted in red. The top view shows the protein with Helix 2 and Helix 3 closest to the viewer. The bottom view shows the RNA binding cleft with Strand A closest to the viewer.
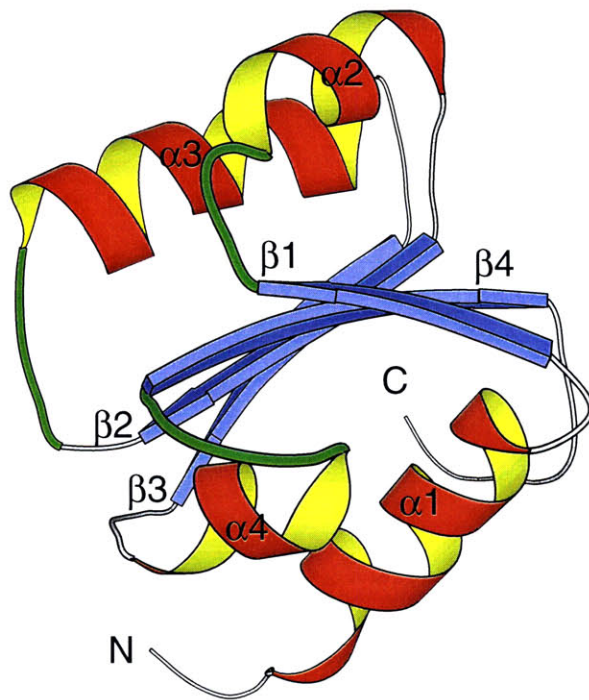
Figure 7.1.2: MOLSCRIPT (Kraulis, 1991) ribbon representation of the L30 protein in the bound form. The N- and C-termini of the protein are labeled, as well as the helix and strand secondary elements. The RNA binding loops are highlighted in green.
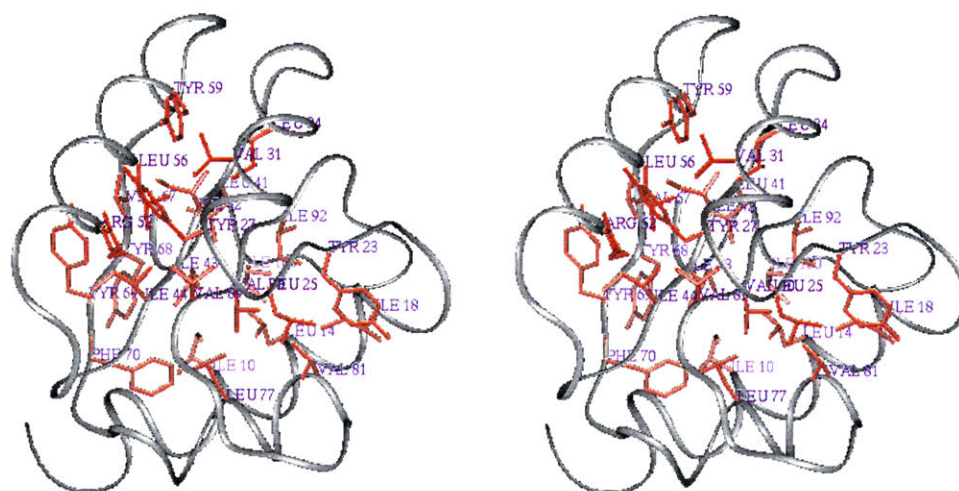
Figure 7.1.3: Stereo view highlighting the hydrophobic core of the L30 protein. The side chains of these residues are shown in red, and the protein backbone is traced in grey.

rate and a crippled of 60S subunit of *S. cerevisiae* upon deletion of Ile43 (Vilardell & Warner, 1997).

Besides those in the β-sheet, conserved hydrophobic residues are also seen within the four helices, which are particularly important in positioning the helices. Among the four helices, residues 10 to 18 from Helix 1 show the best-superimposed backbone and sidechain r.m.s.d. among the ensemble of SA structures. The ordered arrangement of Helix 1 is accomplished by extensive hydrophobic contacts from Helix 1 to one edge (strand C) of the β-sheet to the other one (Strand A). More specifically, residue I10 of Helix 1 interacts with Tyr68 and Phe70 of Strand C define the starting position of this helix. The hydrophobic interactions are further propagated through residue Leu14, which makes contacts with Ile43 of Strand B and Ile92 of Strand D, and terminates with residue Ile18, which interacts with Ile92 in Strand D and Tyr23 in Strand A. The well-defined orientation of Helix 1 is also fortified by the peripheral hydrophobic interactions between Helix 1 and Helix 4, and between Helix 1 and the C-terminus.

The orientations of Helix 2 and Helix 3 are defined through the hydrophobic contacts to the β-sheet, in conjunction with interactions between the two helices. First, the position of Helix 3 with respect to the β-sheet is defined through hydrophobic contacts between Ala60 and Leu56 on Helix 3 to hydrophobic residues of Strand C and Strand B, and fortified by the tight turn connecting Helix 3 and Strand C. As a result, Helix 3 has a very well defined structure, particularly at the C-terminus. In contrast, Helix 2 has only a few interactions with the β-sheet, with only residue Leu34 contacting the β-sheet. The position of Helix 2 is held mainly through helical amphipathic contacts to Helix 3. These interactions can be seen between residues Tyr27, Val31 and Arg35 on Helix 2, and residues Arg52, Leu56 and Tyr59 on Helix 3. Conserved hydrophobic residues Tyr27 and Val31 from Helix 2 intercalate into the grooves formed between Arg52 and Leu56, Leu56 and Tyr59 from Helix 3. These intercalating interactions help to orient the two helices with respect to each other.

Among the four helices, Helix 4, though locally well define as a helix, has the least ordered orientation in the NMR derived ensemble of structures. This is most likely the direct

result of a lack of sufficient long-range distance restraints in the structure calculation, and not because of the dynamical disorder. A few long range contacts were observed in the NMR studies: the side chain of Leu77 on Helix 4 makes hydrophobic contacts to Phe70 of Strand C, Ile43 of Strand B and Val90 of Strand D, and the side chain of Val 81 on Helix 4 also contacts parts of Helix 1. But in general, these long-range contacts are not sufficient to define a high-resolution structure in the NMR-based calculations. The lack of NOEs rather than an intrinsic flexible conformation is also supported by the backbone dynamics of the protein in the complex form, which show comparable relaxation T_1, T_2 and heteronuclear NOE parameters to the other ordered segments of the protein. Therefore, the apparent less-ordered orientation of Helix 4 in the ensemble of structures is expected in the structural calculations.

7.1.1.4. Salt bridges in the protein

While the interior of the L30 protein is mostly hydrophobic, the exterior is more hydrophilic and comprises a reasonable percentage of charged residues. As can be seen in the primary sequence alignment of L30 proteins from several species (see Figure 1.2.1), there are several strictly conserved charged residues within the protein sequences. Detailed analyses of the ensemble of SA structures show that the side chains of these residues (particularly Lys22, Arg35, Lys53, Glu55, Glu57 and Glu94), though partially disorder at the terminal groups since no additional restraints were included for the structure refinement, are reasonably ordered. As a result, the side chains of these residues relative to the remainder of the protein are sufficiently positioned, allowing identification of potential salt bridges present in the protein. Several of these side chains residues were identified to be in close proximity in the ensemble of SA structures, which suggests the presence of salt bridges.

Detail analysis of the structure suggests the presence of the following salt bridges, either within a helix, between two helices, or at a helical turn, thereby further contributing to the protein stability. Among them, an interhelical salt bridge is formed between conserved residues Lys53 and Glu57 in Helix 3, which contributes the helix stability. The remaining salt bridges are found in regions involving the protein packing. Residue Arg35 from Helix 2 and E55 from Helix

3 formed an interhelical salt bridge at the periphery of the two parallel-packed helices, which enhances the hydrophobic packing between Val31 of Helix 2 and Tyr59 of Helix 3. Another salt bridge exists between Lys22 of Strand A and Glu94 of Strand D, which fortify the interactions between Strand A and D, as well as strengthen the turns connecting Helix 1 and Strand A.

7.1.1.5. Functional roles of the glycines

Given the positions of the secondary structure elements, most loops are rather short, and many of them carry glycines. There are a total of nine glycine residues in the yeast L30 protein, seven of which are highly conserved throughout a number of species: Gly21, Gly26, Gly37, Gly71, Gly78, Gly82 and Gly96. Except for Gly78, all the other conserved glycines are located within the loops connecting the α-helices and β-strands. Glycine has been observed to carry the most favorable helix-terminating propensity among all amino acids, therefore the presence of these conserved glycines within the turns are important for making the transitions between regular secondary structures. The only two turns in which glycine is absent are the one connecting Strand B and Helix 3, which uses a conserved proline residue, and the other one connecting Helix 3 and Strand C, which instead uses a tight hydrogen bond between three residues instead.

The loop connecting Strand C and Helix 4 displays significant flexibility, most likely due to the residues Gly72, Gly73, Asn74 and Asn75 within this segment. The apparent flexibility within this region is not only visible from the superimposed SA structures, it was also observed in the protein backbone ^{15}N relaxation studies. The conserved Gly72 has a negative heteronuclear ^{1}H-^{15}N NOE value (-0.19) in the dynamics measurements, suggesting the disorder results from the intrinsic flexibility adopted by this region, but not from insufficient long distance restraints in the structure calculations.

7.1.2. RNA structure

7.1.2.1. Secondary elements observed in the NMR studies

The use of both uniformly and selectively (G-only) $^{13}C/^{15}N$-labeled RNA samples, in combination with four mutant (U14C, A55C, A55U and A57C) studies allowed accurate interpretation of many NOEs within the two helical stems and the asymmetric internal loop of the L30 RNA in the bound form. The sequential imino-imino and nonexchangeable aromatic (H8/H6/H2) to anomeric (H1', H2' and H3') protons connectivities all indicated the existence of two helical regions. The selective labeling and mutant studies provided many essential resonance assignments within the internal loop region, which allowed the accurate identification of an unusual base pair and a unique conformational arrangement of the nucleotides within the internal loop.

7.1.2.2. Overall description of the RNA structure from molecular modeling

The presence of two nearly A-form helical stems joined by a highly distorted internal loop can be clearly seen in the averaged SA structure shown in Figure 7.1.4. The RNA adopts an overall bent conformation, in which the helical conformation unwinds at the internal loop region. The relative orientation of the two helices appears to have an overall 90° bending away from a coaxial stacking conformation, which is a direct result of the asymmetric internal loop and the protein binding. The backbone of the RNA is significantly distorted in the G56-A59 segment, which adopts a hairpin-like structure. With a bent conformation at the internal loop region, partial continuation of base stacking is maintained by G11, A12, A55, G56 and A59 within the internal loop, while A57 and G58 are bulged out of the stacking conformation to make contact with the protein.

7.1.2.3. Helical stems

As can be seen in Figure 7.1.4, the two helical regions have nearly A-form conformations, as more than three base pairs within each helix adopt a geometry that satisfies A-
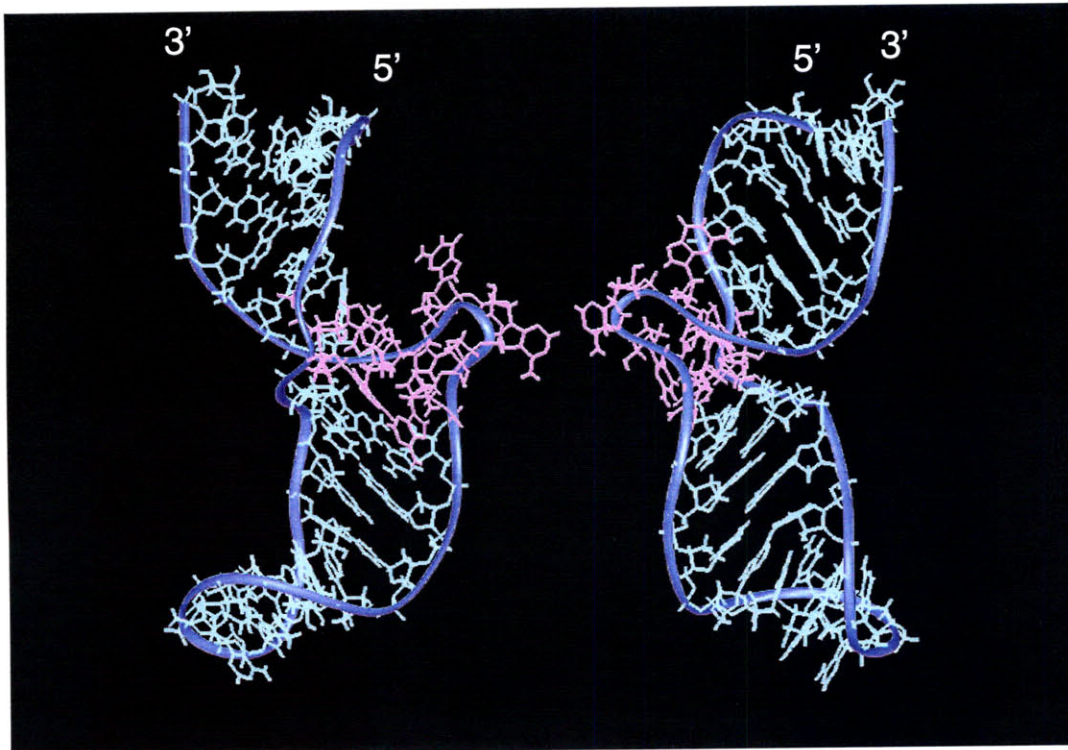
Figure 7.1.4: Two views of the L30N RNA in the bound form. The internal loop is colored magenta. The left view shows the major groove of the internal loop, while the right view shows the minor groove.

form criteria. In a standard A-form RNA, the twist angle for each base is ~33° (verses ~35° for B

form), the inclination is larger than 0° (but ~0° for B) and displacement from the helix axis is ~4

Å (versus ~0 Å for B form; Saenger, 1984). As can be seen in the average structure of the L30N

RNA, most Watson-Crick base pairs with the lower stem (displayed upside down in the figure)

maintain good stacking with an average 30° twist angle, 2° inclination, and 4 Å displacement

from the helix axis. In the upper stem (displayed in the lower part of the figure), the presence of

the U14·G53 wobble base pair appears to distort the A-form helical conformation slightly. This

wobble base pair is nearly unstacked from the base pair 5' to the guanine (C52), but does stack

well on the pair to the 3' side of the guanine G13-(C54). This stacking arrangement leads to a

high twist at the G13-C54 and U14·G53 pair step, but a low twist at the U14·G53 and G15-C52

step. This type of stacking conformation associated with the wobble U·G pair is expected

(Saenger, 1984), and also has been observed in other cases (Allain & Varani, 1995). In addition

to A-form base stacking, many sugar puckers of the residues in both helical regions maintain

C2'-endo conformations, this is also a characteristic of an A-form RNA structure. The only

exception is the terminal G5 with an inter-converting pucker between C2'- and C3'-endo, which

is expected for a terminal nucleotide. While the local conformation of each stem is well defined

(~1.0 Å r.m.s.d. by superposition of each segment), the orientation of one stem with respect to

the other is not as clearly preserved in the superimposed SA structures, as both helices have

about 2.0 Å r.m.s.d. for all heavy atoms in the complex structures. The swinging of the two

helices in the ensemble of structures is mostly due to the lack of long-range distance restraints.

Biochemical analyses have shown that the base identities within the two helical stems are

unimportant for L30 protein binding (Li *et al.*, 1995; Li & White, 1997). However, the

maintenance of a minimum of two to three base pairs within each stem is necessary for the RNA

binding to the protein (Li & White, 1997). The variation in length and lack of sequence

specificity of both helical stems suggests that bases in the stems do not interact directly with the

protein in the complex, but the base pairs within each stem are important in indirectly stabilizing

the RNA-protein interactions. Among the base pairs within the helical region, G10·U60 base

pair adjacent to the internal loop has been studied extensively (White & Li, 1995). While the

G·U wobble base pair is highly favorable for the MBP-L30 fusion protein binding, other non-

standard base pairs also display moderate protein binding affinity. A Watson-Crick base pair is not favorable at this position. This observation is consistent with the NMR structure, as the 10:60 position is juxtaposed to the internal loop and partially unstacks away from the internal loop on the U60 side. Any Watson-Crick base pair in this position would be deleterious to the protein binding, as it would tend to close the major groove that is important for protein binding.

7.1.2.4. Tetraloop

The GCAA tetraloop sequence in the L30N RNA is a result of a sequence modification that was used to stabilize the hairpin structure in the upper stem. Nevertheless, the observed structural features in this study are consistent with previously reported GNRA tetraloop-types of structures (Heus & Pardi, 1991b;Battiste, 1996). The conformation of the GCAA tetraloop in the L30N RNA will be described briefly as follows.

The hydrogen bonding pattern of the shear G18·A21 base pair within the tetraloop is schematically shown in Figure 7.1.5, panel **a**. Two interbase hydrogen bonds are formed in this base pair: one from a G18 exocyclic amino proton to the A21 N-7, and the other from an A21 exocyclic amino proton to the G18 N3. Although no direct NOE evidence was observed for the formation of these two hydrogen bonds, indirect NOEs suggest the formation of a shared G·A base pair. More specifically, through spin diffusion from the G18 amino proton, there is a weak NOE between the G18 imino and A21 H8 protons. In addition, the observed sugar puckers for the nucleotides in the tetraloop also suggest the reversal of the phosphate backbone at the tetraloop region. In the structure, one of the G18 amino protons is within standard hydrogen bonding distance of the A20 N7, but both A21 amino protons are farther away from the G18 N2. However, one of the A21 amino protons is within hydrogen bonding distance of the sugar 2' oxygen on G18. This interaction may help to stabilize the formation of this G·A base pair. To accommodate this unusual G·A base pair, the helical twist for this base pair is small compared to a standard A- or B-form helix. This conformation for A21 is created by a G18 β-torsion angle that is gauche instead of the normal trans. In this conformation there is a large ring current effect of the A21 base on the G22 H1', which accounts for the unusual upfield chemical shift (4.10

a.

G18 : A21

b.
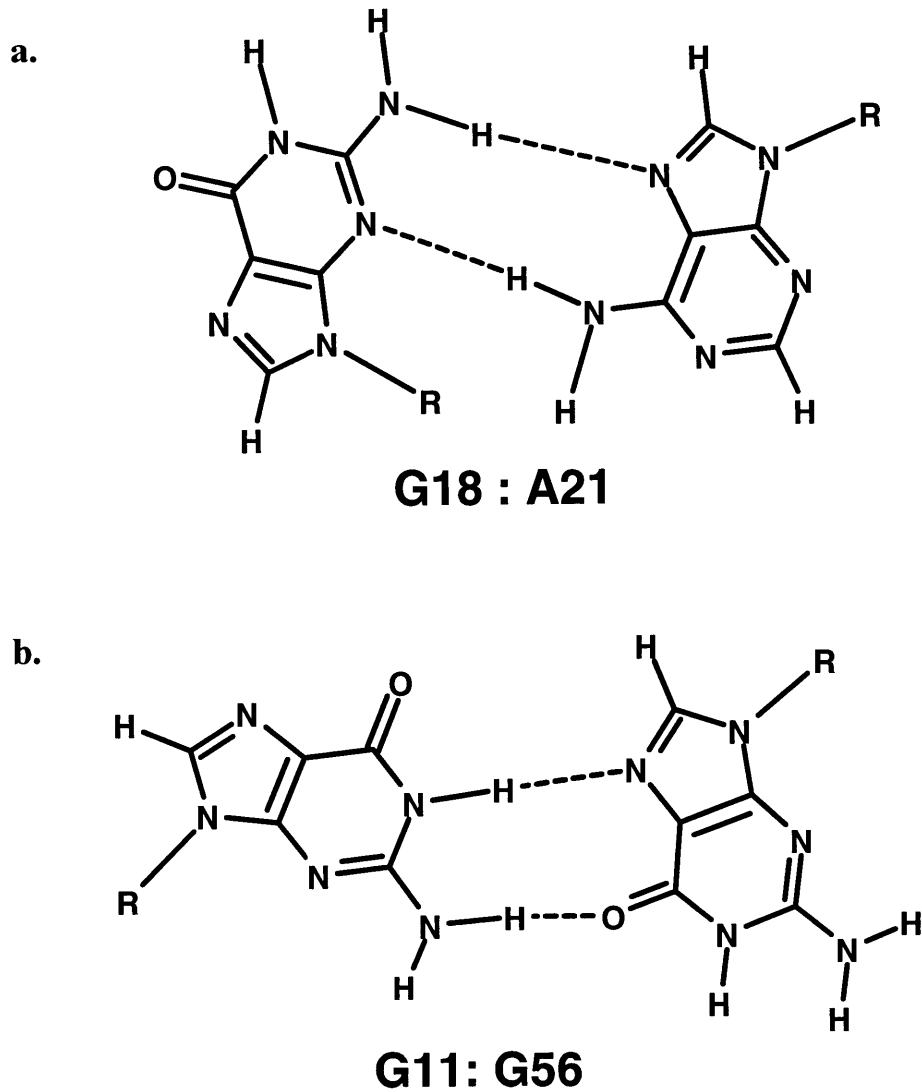
G11: G56

Figure 7.1.5: The sheared G18·A21 pair (top) and reverse Hoogsteen G11:G56 pair (bottom).

ppm) for this resonance. Stacking of the bases in the loop is performed by A21 on G18 and A20 stacking on A8, but C19 does not stack on either G18, A20 or A21. Instead, the sugar phosphate backbone between G18 and C19 is extended, putting the C19 base on top of the hairpin loop. This conformation is supported by internucleotide H1'/H2'-H6/H8 NOEs observed between C19 and A20, A20 and A21, but not between G18 and C19.

7.1.2.5. Internal loop

The purine-rich internal loop is the most important part of the RNA, where the recognition of the RNA by protein takes place. An asymmetric two-plus-five length requirement and sequence conservation for bases at a majority of positions suggest that both the overall shape of the internal loop and the identity of individual bases are important for protein binding. The NMR structure provides an opportunity for observing the base pairing and spatial orientation of the nucleotides in the internal loop, therefore leading to the understanding of the L30 RNA-protein sequence-specific recognition.

As can be seen in Figure 7.1.4 (in which showing the view of the RNA is upside down with 5'- and 3'-end on the top), the phosphate backbones are significantly distorted on both sides of the internal loop. The distorted backbones are facilitated by two distinct conformational arrangements in the internal loop, best described as extensive purine-purine base stackings and a hairpin-type looping structure that reverses the direction of the phosphate backbone. In the first case, purine-base stackings are observed between G11, A12, G56 and A59, with the complete base stacking of A59 on top of G56, and partial stacking of A12 with G11 (Figure 7.1.6). The reverse Hoogsteen base pair between G11 and G56 appears to be the centerpiece of the stacking interactions. Two interbase hydrogen bonds are formed in this base pair: one from a G11 exocyclic amino proton to the G56 O6, and the other from a G11 imino proton to the G56 N7 (Figure 7.1.5). These hydrogen-bonding patterns are supported by observation of the strong NOE between G11 imino to G56 H8 protons. In addition, the *syn* glycosidic conformation and the reversal of the sugar orientation of G56 facilitate the formation of this G11·G56 base pair. Aside from the stabilization of the RNA internal loop through hydrogen bonding, the G11G56

mismatch pair also serves as a platform bridging the protein and RNA together through aromatic stacking (Figure 7.1.6).

During the structure calculation the orientation of A12 within the internal loop has been observed as two major conformers, with 50% of the initial RNA structures in an "out" A12 conformation Figure 7.1.6 in respective panel **a**, and 25% in an "in" A12 conformation (Figure 7.1.6, and panel **b**). The so called "in" and "out" refer to the position of A12 with respect to the G11·G56 base pair, where "in" refers to A12 stacking between nucleotide A59 and the G11·G56 base pair, and "out" refers to stacking outside and below the G11·G56 pair. As can been seen in a superposition of the average structures of the two conformers (shown in Figure 7.1.7), most of the remainder of the residues adopt very defined structures in the two conformation; only the relative position of A12 is not defined. These observations are a direct result of insufficient long distance restraints for this nucleotide in the molecular modeling, with no significant overall energy or NOE violations were observed between these two conformers. However, NMR observations and detailed structural analyses suggest that the "out" conformation could likely be the actual position adopted by A12. More specifically, the strong G11 imino proton resonance has been observed upon complex formation, indicating potential hydrogen-bond formation. In addition, a medium-strong NOE was observed between the G56 imino proton and the A12 H2 proton. Furthermore, the A12 was found in *in vitro* selection studies to be a conserved residue for protein binding (Li & White, 1997). In the modeled structure, this imino proton is very close to the A12 N1 in the "out" conformation, but not close to other hydrogen acceptors in the "in" conformation. This observation greatly suggests the possibility of hydrogen bonding formation between G56 imino proton and A12 N1. As a result of this hydrogen bonding arrangement, no base pair is formed between A12 and A55 (which was initially anticipated). In the structure, A55 is fully stacked above the G13-C54 base pair, and A12 slides on top of A55 for hydrogen bonding with G56. The absence of base pair arrangement between position 12 and 55 is supported by experimental observations. First, both A12 and A55 adopt *anti* glycosidic angles, whereas a *syn* angle is expected in an A-A base pair. While sequential stacking NOEs are observed between A55 to the G13-C54 base pair, very weak or no NOEs are observed from the A12 base to the G13-C54 base pair. Furthermore, the adenine at position 55 is not conserved and
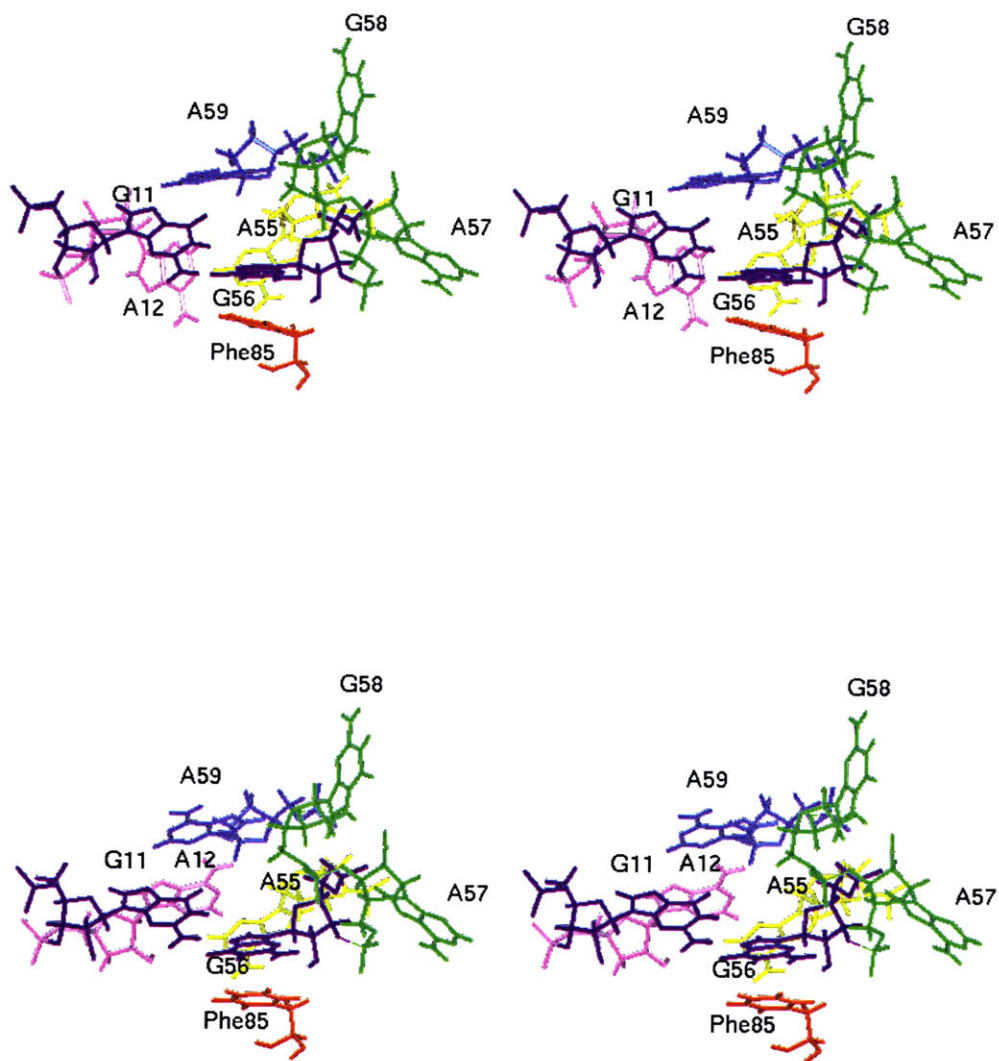
Figure 7.1.6: Stereo views of the internal loop of the L30N RNA. Both panels show the continuous stacking of Phe85, G56, and A59 with residues A57 and G58 looping out of the RNA helix. In the top panel, A12 ("out" conformation) is below G11, while in the bottom panel residue A12 ("in" conformation) is stacking between G11 and A59.
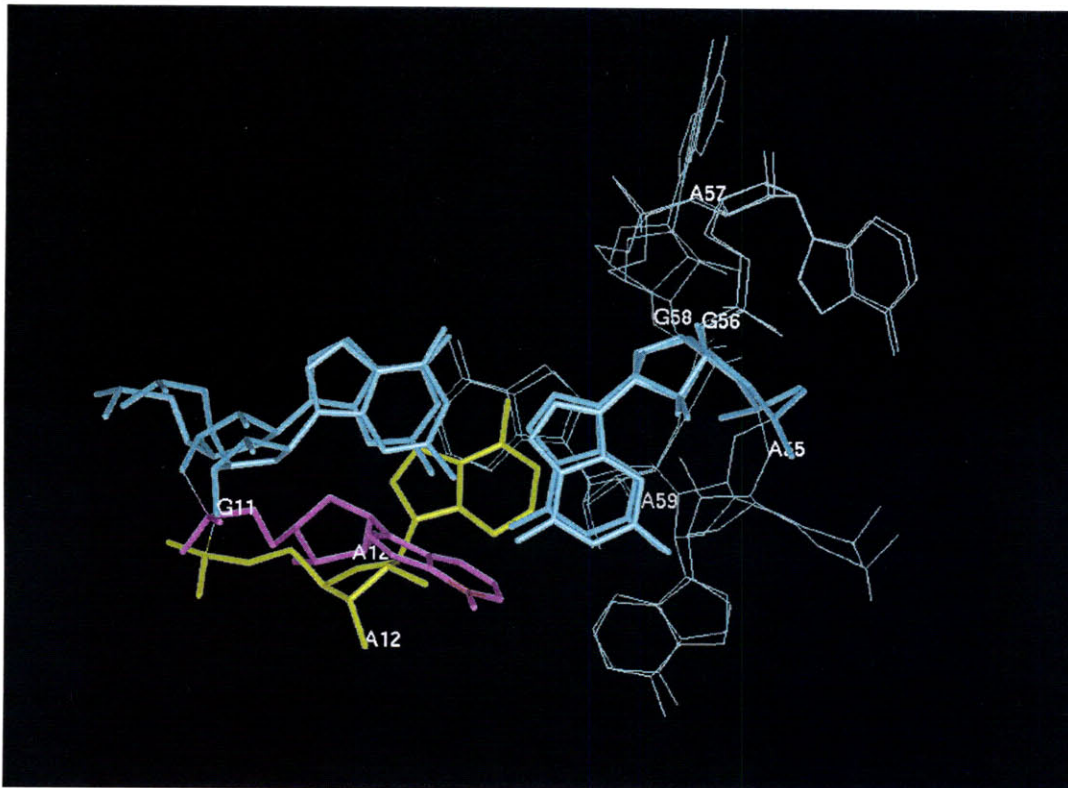
Figure 7.1.7: Superposition of the average structures of two conformers showing the internal loop of the L30N RNA. The "out" conformation of residue A12 is in magenta, and the "in" conformation of residue A12 is in yellow. The G11-G56 Hoogsteen base pair (in blue) is emphasized.

can substituted with other nucleotides. Substitution of A55 to a uracil could potentially create a base pair between A12 and U55. However, the A55U mutant shows no observable uracil imino resonance that would be expected in a base-paired conformation.

Besides the stacking interaction maintained by most residues within the internal loop, A57 and G58 are bulged out of the helical region to make base-specific contacts with the protein. The bulge conformations were anticipated from the NMR observations, since these two nucleotides lack both interresidue and interstrand stacking NOEs to the rest of the nucleotides in the RNA, but show overwhelming NOEs to the protein. Despite the lack of intra-molecular hydrogen bonds with residues in the internal loop, each of the unpaired nucleotides is involved in intermolecular hydrogen bonding and hydrophobic interaction with the protein. As a result of the bulge conformation of A57 and G58, and the resumption of base stacking in A59, the phosphate backbone of the RNA is dramatically kinked in this region. The conformations adopted by these two nucleotides create a dramatic distortion at the phosphate backbone, since both bases are protruding into the concavity formed by the protein loops. The appearance of the hairpin like phosphate backbone is supported by the observation of the long range NOEs between the G56 H4' to base and sugar protons of A57 and G59. This conformation can also be explained by the observation of medium-strong NOEs between the H1' protons of A59, G12 and A55.

7.1.3. Specific intermolecular contacts

Figure 7.1.8 shows render surfaces of two views of the L30 protein in the bound form. One face of the protein carries a large concavity with distinctive electropositive residues on the sides, in which one side of the RNA internal loop makes contacts with protein. As a result of the conformational arrangement in the RNA internal loop, two distinct clusters of protein residues involved RNA recognition are observed in the complex structure. One cluster is centered on base G56, with which contacts are made from the conserved residues Leu25 and Gly26 from Strand A, Phe85 and Val87 from the loop connecting Helix 4 and Strand D. The other one is the concavity formed between two loops that contact bases A57 and G58, involving residues Tyr27

and Ly28 from the loop connecting Strand A and Helix 2, Asn47, Thr48, Pro49 within the loop

connecting Strand B and Helix 3, and Arg52 in Helix3. The RNA-binding site in the L30 protein

involves a combination of conserved basic residues, and conserved and solvent-exposed

hydrophobic residues.

In the absence of RNA binding, there are two stretches of amide protons that display

significant exchange broadening. The shorter one is from Tyr27 to Thr30, corresponding to the

loop region connecting Strand A and Helix 2. The longer one involves residues Asn74 to Gly88,

corresponding to Helix 4 and the loop connecting it to Strand D. Upon binding of the RNA,

these residues became much more ordered, judging from the sharpness of the resulting amide

proton resonances and extensive NOEs from the side chain of these residues. From the NMR-

derived structure, many of these residues participate in the RNA binding interaction (Figure

7.1.9).

7.1.3.1. Hydrophobic contacts

While the helical conformation is extended into the internal loop by the complete

stacking of A55 above the G13-C54 pair, a change in the stacking direction occurs between A55

and G56. The reverse Hoogsteen mismatch base pair formed between G11 and G56 orients

almost orthogonal to residue A55. The stabilization of this conformational arrangement not only

comes from the base stacking and hydrogen bonding within the bases in the internal loop, it also

is a direct result of hydrophobic interaction from the protein. These interactions involves

conserved residues Leu25 and Gly26 from the edge of Strand A, and Phe85 and Val87 from the

loop connecting Helix 4 and Strand D. Hydrophobic residues Leu25, Phe85 and Val87 form a

hydrophobic, concave surface around G56, and heightened by an almost complete stacking

interaction of Phe85 with G56 (Figure 7.1.10, panel **a**). The aromatic ring of Phe85 acts like a

shim that protrudes into the two bases, resulting in the changing of the base G56 orientation with

respect to A55.
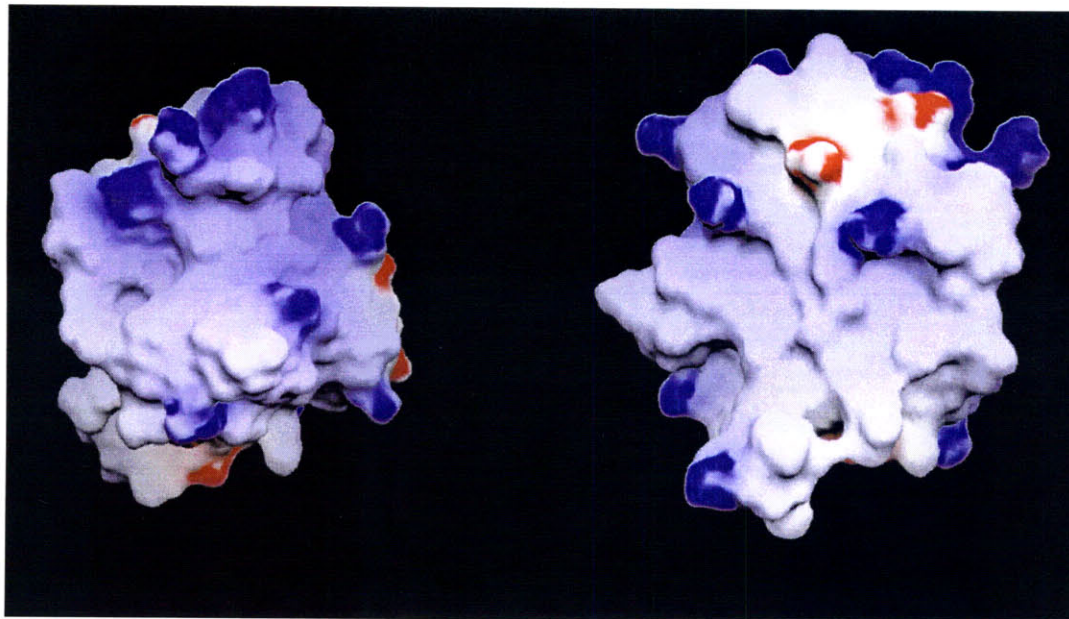
Figure 7.1.8: Molecular surface of the L30 protein in the bound form showing the electrostatic surface contour from -10 k_BT (red) to +10 k_BT (blue). The electrostatic surface was calculated by the program GRASP (Nichols *et al.*, 1991). On the left is the RNA binding cleft which has a high density of positively charged residues. The right side shows the opposite face of the protein.
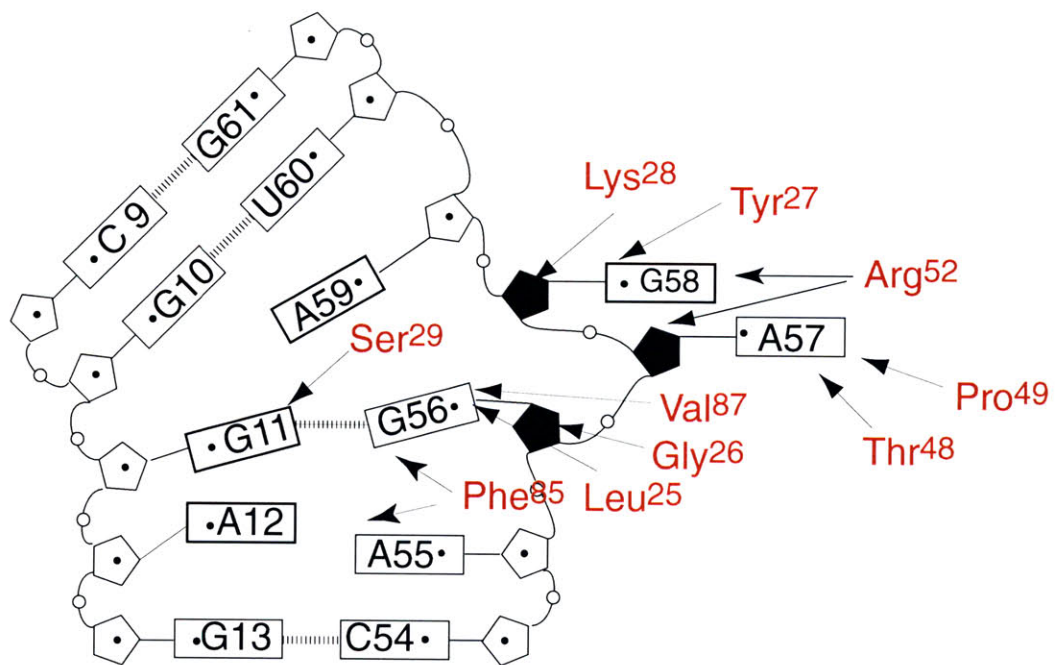
Figure 7.1.9: Schematic representation of the protein-RNA NOEs.
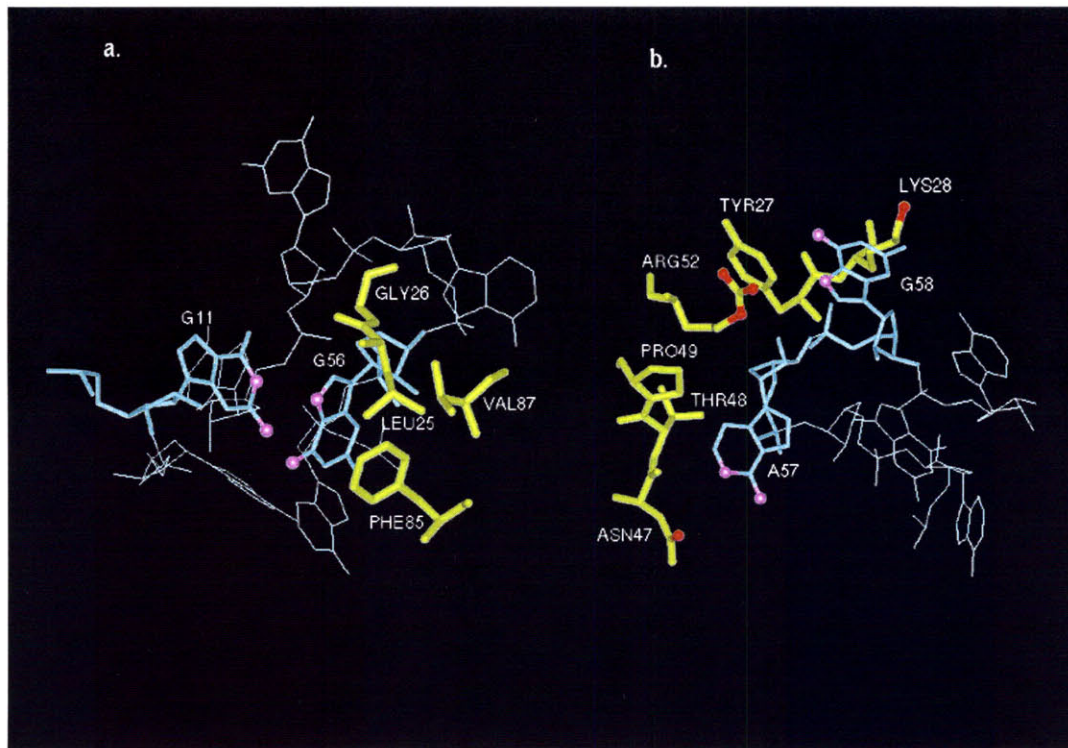
Figure 7.1.10: Contacts between the protein (yellow) and RNA (cyan) in the L30 complex. Panel **a** shows the hydrophobic contacts between Leu25, Phe85, and Val87 on the protein and G56 on the RNA. Panel **b** shows the specific recognition of Asn47 with A57 and Arg52 with G58.

Another hydrophobic contact involves Pro49 and A57. It is likely that Pro49 serves to turn the amino acids that lead Strand B into Helix 3, the hydrophobic side chain could also be responsible for stabilization of the conformation of A57.

7.1.3.2. Specific recognition

Base specific recognition between protein and RNA, on the other hand, plays major roles in stabilization of the conformations of the internal loop. Examples of this type of interaction include the observation of two base-specific recognitions (Figure 7.1.10, panel **b**). A detailed close-up view shows that Arg 52 is contacting G58, Asp 47 with A57, and Lys 28 with either the base or the phosphate of A59. G58 is stabilized by a hydrogen bonding interaction between the G57 O6, the G57 N7 and the guanidinium group from Arg 52. Hydrogen bonds are formed between the A57 H61 and the Asn47 carboxyl group, and A57 N7 and the Asn47 amino group. These interactions are further fortified by the hydrophobic interaction between the A57 aromatic ring and Pro49.

In many protein-nucleic acid interactions, arginines and asparagines play critical roles in recognition (Mandel-Gutfreund & Margalit, 1998). In the case of arginine, two types of interactions involving arginines have been found. The first type is the direction hydrogen bonding interaction between the guanidinium group of an arginine and the O6 and N7 atoms of a guanine (Cai *et al.*, 1998; Battiste, 1996; Elrod-Erickson *et al.*, 1998). With three lone pairs of electrons (two from O6 and one from N7), guanine is the best nucleotide for accepting hydrogen from the guanidinium group of arginine. The second type of interaction uses an arginine to neutralize negative charges on the phosphate backbone on complex formation (Battiste, 1996). While arginines are always associated with base specific recognition of guanines, asparagines have been observed to interact with adenosine and cytosine. The side chain of an asparagine serves as both hydrogen-bond donor and acceptor, which interact preferably with adenine and/or cytosine at the Watson-Crick face. Taking these into account, it is obvious that residues Asn47 and Arg52 in the L30 protein are conserved throughout all species, while A (or C), and G are most frequently observed in position 57 and 58 respectively.

7.1.3.3. Other possible contacts

In addition to the above interactions and specific contacts, analysis of the complex structure also suggests other possible contacts between the protein and RNA. Looking into the RNA binding surface on the protein, Helix 2 is particularly in close proximity to the RNA. More specifically, a majority of residues at the N-terminus and one face of Helix 2 are involved in the intermolecular contacts. Among them, three residues Lys28, Ser29 and Lys32 could play major roles in either forming hydrogen bond or making phosphate backbone contacts to the RNA. Briefly, significant numbers of NOEs were observed from the side chain and backbone protons of Lys28 to the sugar and base protons of G56 on the RNA. In the modeled structure, the side chain of Lys28 is in parallel position next to G56 base, which appears to involve in hydrophobic stacking interaction with G56. On the other hand, the positively charged side chain of Lys28 could potentially involve in making backbone contact to the phosphate of A56. Second, the NOE observations and the structure modeling suggest that the hydroxyl group of Ser29 may involve in a long-range hydrogen bond contacts with two RNA bases. The hydroxyl group of Ser can serve as either hydrogen bond donor or acceptor. As a donor, the hydrogen on the hydroxyl group is near two potential hydrogen-bond acceptors: the O6 of G or N7 of G11. As an acceptor, its oxygen can potentially form a hydrogen bond with the exocyclic amino group from the A57 in the RNA internal loop. It is also possible that the S29 may also form a water-mediated hydrogen bond with purine bases; this type of hydrogen bond is frequently observed in the X-ray crystal structures of protein-DNA complexes (Elrod-Erickson *et al.*, 1998). An additional interaction from Helix 2 is observed at residue Lys32, which is positioned on the side of Helix 2 as Ser29. Throughout the complex structural calculation, the side chain of Lys32 is in close proximity to the phosphate backbone of G10. No direct NOEs were observed between the side chain protons of Lys32 and the ribose protons of G10; this may suggest that Lys32 be involved in non-specific charge contact with the phosphate backbone.

7.2. Structural Similarity of L30 with Other Proteins

Structures of a number of RNA-binding proteins have been determined, with most of them belonging to the αβ family protein. These include the U1 snRNP protein A, aminoacyl-tRNA synthetases, many prokaryotic ribosomal protein (*e.g.* S5, S6, L1, L6, L7/L12, L9, L22 and L30) and proteins from RNA viruses (*e.g.*, tobacco mosaic virus, and bacteriophage MS2 protein), which carry an **abc/αd**-unit with a split β-α-β motif. The schematic presentation of the basic **abc/αd**-unit (with two variants) is shown in Figure 7.2.1, in which region **c** has an α-helical conformation, regions **b**, **c** and **d** form the right-handed β-α-β superhelix, and strand **a** is located between strands **b** and **d** in the direction antiparallel to them (Rao & Rossmann, 1973). Variants are also observed with the insertion of other secondary structures within each unit, but a left-handed crossover is generally prohibited (Richardson *et al.*, 1992).

The structure of the L30 protein has been compared with known RNA-binding proteins to look for the evolutionary relationships and common RNA-binding motifs. To search for protein structures that are homologous to L30, the coordinates were submitted to the program SCOP (Murzin *et al.*, 1995). Currently, the structure of the L30 protein is not homologous with any of the known protein structures. The L30 protein presents a novel topology with a β-sheet formed by a mixture of parallel and anti-parallel β-strands sandwiched between two sets of helices. The arrangement of three anti-parallel strands and one parallel strand β-sheet has been observed in RNase P (Stams *et al.*, 1998), ribosomal protein S5 and ribosomal translocase elongation factor G. However, close structural inspection suggests that these proteins, with the unusual left-handed β-α-β crossover topology, are different from the L30 protein. Detailed analysis of the L30 folding topology shows that the protein exhibits a right handed β-α-β crossover, which is closely related to the **abc/αd** family shared by many other RNA binding proteins. For instance, Strand D, Strand B, Helix 2 and Strand A can be considered as the respective **a**, **b**, **c** and **d** regions within the **abc/αd**-unit, while Helix 3, Strand C and Helix 4 can be regarded as a large insertion between **a** and **b** regions (See Figure 7.2.1 (3)). The long loop connecting **ab** regions has been observed in the structure of chorismate mutase (Chook *et al.*, 1993). While only

(1)

(2)

(3)
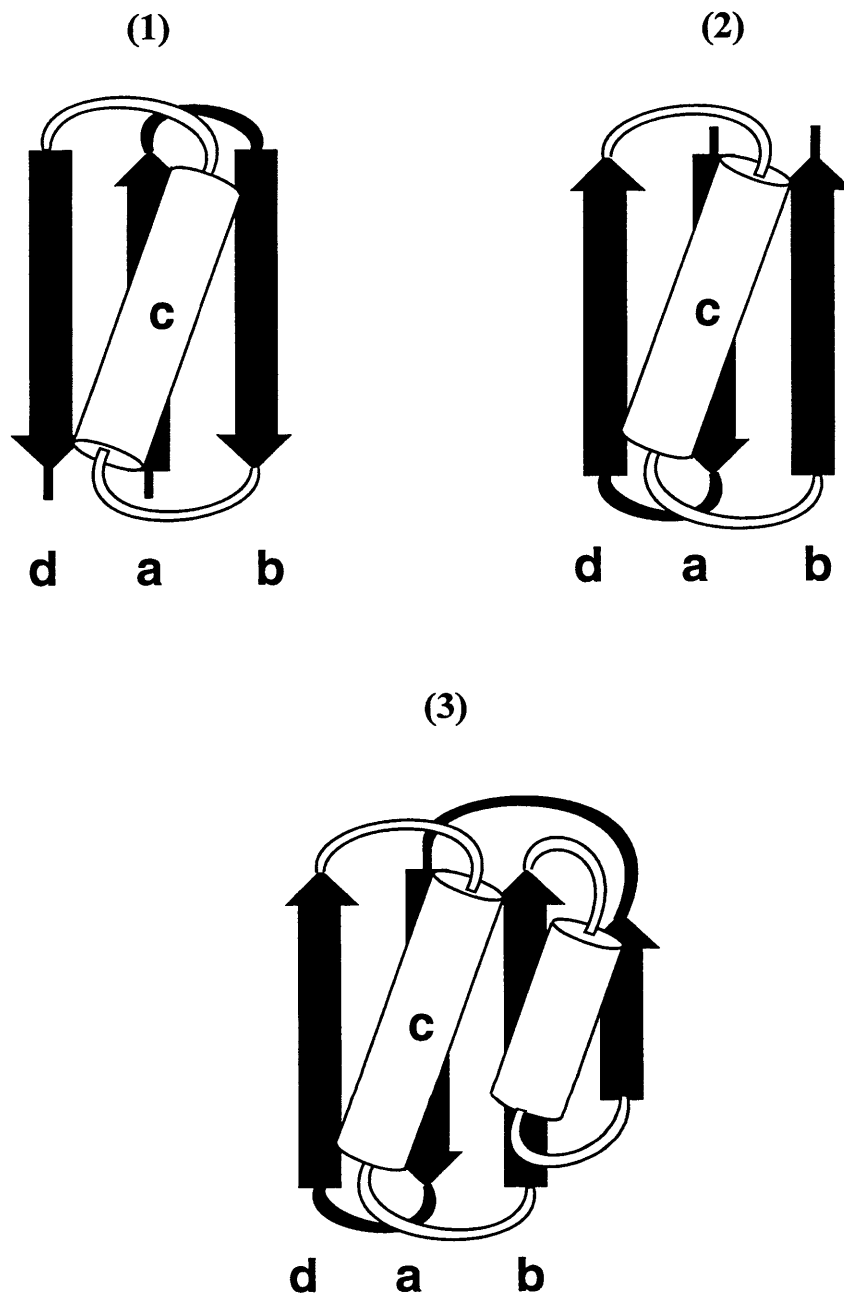
Figure 7.2.1: Schematic representation of the basic **abc/αd**-unit (1) and (2), and one of the variants (3) with a long loop between the **a** and **b** segments.

considering the segment of the **abcd** region, segments of four other proteins are found to share a close similarity with L30 protein, including the N-terminal domain of S8 (Davies *et al.*, 1996), DNase I (Weston *et al.*, 1992), DNA methyltransferase (Reinisch *et al.*, 1995) and EPSP synthase (Stalling & Kishore, 1991).

7.3. Biological Implications:

7.3.1. Correlation with biochemistry data:

The impact of the various base specific interactions with L30 protein in stabilizing the L30 protein and RNA complex has been demonstrated by biochemical mapping and SELEX experiments (Li *et al.*, 1995; Li & White, 1997; White *et al.*, 1995). In contrast to testing individual point mutants that might affect the complex stability, SELEX experiments showed an accelerated *in vitro* phylogenetic conservation of the important bases in the binding, which was consistent with the chemical modification interference results. Backbone modification of A55 and G56 with ENU abolishes protein binding. Carboxyethylation of all purines except A57 in the internal loop either abolished or strongly inhibited the binding.

Conservation of G11 in SELEX studies and inhibition of binding by carboxyethylation at this position can be explained by hydrogen bonding recognition from Ser29. Specifically, the hydroxyl group from the Ser29 side chain forms a hydrogen bond with the G11 N7 (or O6) position. In addition, G11 also forms a mismatch base pair with G56, using the Watson-Crick face to hydrogen bond with G56. The G11 to C substitution reduces the binding by about 7-fold (data from S. A. White).

The functional importance of A12 might lie in the stacking potential in stabilizing the complex structure. Among the four nucleotides, adenosine provides the strongest stacking stabilization, and purines in general are better than pyrimidines (Saenger, 1984). The absence of a G substitution at position 12 could be explained by the steric hindrance and electrostatic

repulsion arising from the presence of the bulky exocyclic 2-NH$_2$ group in G which replaces the H2 proton of A in the minor groove. Consistent with the observation that the close proximity of H1' protons of A59 to A55 on the same strand and G13 on the cross strand leaves little room for the amino group.

In the SELEX experiment, A55 is the least conserved residue, which can be substituted with either C or U. Binding constants measured by gel shift assay with either RNA replacement are almost identical to wild type RNA (data in Chapter 2). Interestingly, a deletion study showed an absolute necessity of this nucleotide, since removal of A55 completely abolishes the protein binding (White *et al.*, 1995). The functional importance of the 55 position may lie in the phosphate backbone and spacing between the internal loop bases and the upper stem. Consistent with the abolished binding by ENU modification of A55, its role might explain the observation of a conserved Arg86 close to the phosphate backbone in the NMR structure. Arg86 in this case serves to neutralize negative charges on the phosphate backbone and bring the RNA close to the protein.

Conservation of G56 is obvious from the NMR refined structure, since it not only forms hydrogen bonds with G11 using both the N7 and O6 positions, it also makes extensive contacts with protein. Specifically, there is a hydrophobic core made up by Leu25, Phe85 and Val87 surrounding G56, and this is accentuated by the complete aromatic stacking of Phe85 under G56. Consistent with biochemistry data, carboxyethylation of N7 positions strongly inhibit protein binding and ENU modification of the phosphate abolishes protein binding. In addition, substitution of G56 with C greatly reduces the binding affinity to L30 protein (> 300 fold). Concomitant with the stacking observation, replacing the Phe85 with an Ala completely abolishes the protein binding to the wild type RNA (> 1000 fold).

The observation of an unusual frequency of C substitution at the A57 position could be explained by the similarity of the Watson-Crick faces of cytosine and adenine. Both could potentially form two hydrogen bonds between the carboxyamide of Asn47, specifically that between the Oδ1 atom of Asn and the exocyclic amino group of either A or C, and the NHδ

group of Asn to the N1 or N3 of the respective A or C. This is consistent with the observation that Asn47 is phylogenetically conserved among various species in the ribosomal protein L30.

The importance of G58 can be rationalized by the recognition of Arg52. Direct hydrogen bonding interactions between the guanidinium group of arginine and the O6 and N7 atoms of a guanine stabilizes this specific recognition. With three lone pairs of electrons (two from O6 and one from N7), a guanine is the best nucleotide for accepting hydrogen from the guanidinium group of an arginine. Upon carboxyethylation of G58, the bulky ethyl group not only serves as steric interference with Arg recognition, but also prevents the formation of proper hydrogen bond involving the N7 position.

The conservation of A59 in SELEX experiments, and the carboxyethylation at A59 interfering with protein binding can be explained by the recognition by Lys28. Although no direct NOE was observed from the A59 H8 to Lys28, the direct penetration of Lys28 into the major groove and targeted towards A59 could be easily seen in the refined structure. There is a potential hydrogen bond formation from the side chain amino group towards the A59 N7 position, consistent with the observation that substitution of Lys28 with Ala reduces the binding by at least 20-fold (data from S. A. White).

The functional importance of theG10·U60 base pair is less understood from the structure. In the NMR analysis, while a wobbled G·U pair in the free RNA was observed at low temperature (<10°C), this base pair (in particular U60) in the complex underwent chemical exchange that broadened out the resonance. Although biochemistry data indicate that G·A, C·C, U·A, U·C or G·G can replace this base pair with lower binding affinity and a Watson-Crick G-C base pair is highly unfavorable, no direct NOE was observed to either bases. A potential contact might come from the conserved Lys32 that is close to the phosphate of G10 in the NMR refined structure. The stabilization of the binding comes from the charge neutralization rather than specific base recognition.

7.3.2. Relationship to splicing

The ribosome is the site of protein synthesis in all organisms. It is a large, two-subunit complex of three RNAs and up to 75 proteins in most eukaryotic organisms. The regulation of ribosomal protein synthesis is an important step in controlling the production of the ribosome assemblage. The model of autoregulation specifies that binding of L30 prevents the splicing of the RPL30 transcripts. The molecular model predicts that the MBP-L30 fusion protein would not affect the splicing of the mutant transcripts of RPL30 to which it does not bind, which was confirmed by the C9U mutant result. In the splicing, the first step appears to be the binding of the U1 snRNP to the transcript, probably at the 5' splice site to form a commitment complex. Subsequently, in an ATP-dependent process, several splicing factors as well as the U2 snRNP bind, followed by the assembly of other components to form the complete spliceosome.

The co-existence of U1 snRNP and L30 protein on the pre-mRNA suggests that the binding of L30 to its transcript blocks splicing at a step before the association with the U2 snRNP (Vilardell & Warner, 1994). It seems likely that the U1 RNA displaces nucleotides 6-9 (GACC) from their base-pairings with the 5' splice site without eliminating the binding of L30. As can be seen in the structure of the L30 protein-RNA complex, the L30 protein should be in close proximity to the U1 snRNP. However, the displacement of the L30 protein away from the potential U1 snRNP binding site (nucleotide G61 and beyond) in the complex could potentially allow base pairing without significant steric interference. Furthermore, the L30 protein might also interact with part of the U1 snRNP that further stabilizes the nonspliceable complex, therefore prevent in the association of the U2 snRNP that is necessary to form a complete spliceosome.

```
                                        splice site

                                             ⇩
         RPL30    3'  ...  U  A  U  G  A  C  U  G  G  ...    5'
                           |  |  |  |  |     |  |  |
         U1   m3Gppp  A  U  A  C  U  U  A  C  C  ...    3'
```
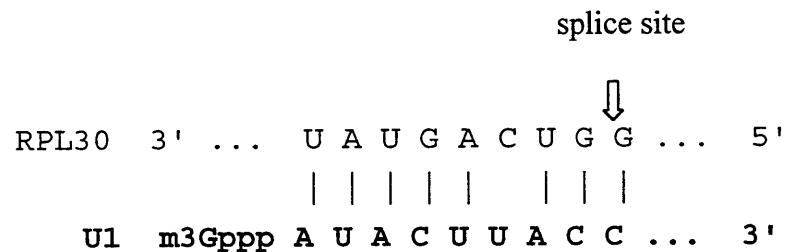
7.3.3. Relation to the yeast ribosome

The ribosome is the center of protein synthesis in all cells, and for over 30 years many efforts to understand its structure and mechanism have been ongoing. Such a goal represents a significant challenge because of the complexity involved in the molecule. The contribution here towards that aim is the determination of the structure of the important eukaryotic ribosomal protein L30. The overall structural features and specific functional residues with its cognate auto-regulatory binding site might reveal a potential ribosomal RNA binding site, which can be correlated to the ongoing research in this area. The L30 protein structure also facilitates further experiments to be designed to probe the local ribosomal environment that can associate with L30 protein, and further assists in the understanding of the function of an eukaryotic ribosome through reconstruction.

7.4. Future Research

7.4.1. Protein mutants

At present, the role of the RNA in the autoregulation has been studied extensively in many biochemical analyses. A 2 + 5 asymmetric internal loop and sequence specific conservation of several purine residues are the most important recognition elements on the RNA side of the complex. In contrast, few biochemistry studies have been carried out in understanding the functional role of the L30 protein in recognition. The L30 protein-RNA complex structure presents a vivid picture describing in detail the possible structural elements that might be important for recognition. However, these structural interpretations can only be validated with confirmation from biochemistry studies.

Currently, an ongoing mutagenesis analysis of L30 protein is being carried out by a co-worker in this laboratory, based on the functional elements present in the NMR-derived complex

structure. These include the replacements of Leu25, Tyr28, Lys29, Asn47, Arg52, Phe85 and Arg86 with alanine to study the specific interactions (*i.e.*, base-specific recognition, hydrophobic interaction) interpreted from the NMR structure. These mutation studies are primarily based on the gel shift binding assay to derive the binding constants. However, gel shift experiments can only provide limited information on the protein recognition, namely binding or no binding. Information including the preservation of the protein tertiary structure and binding constants >μM is difficult to obtain in this experiment. Future research can be carried out using relatively simple NMR (*e.g.*, ^{1}H-^{15}N-HSQC experiments) to analyze the tertiary structure and poor binding constants. In addition, calorimetry titration and denaturant unfolding of the complex can also be used to explore the stability of the complex.

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In addition to residue specific mutagenesis, a phage-display method can be used to screen potential RNA recognition elements within the protein. The phage display selection has provided much useful information regarding the DNA-binding specificity in a zinc finger-DNA complex (Rebar & Pabo, 1994). In principle, the gene of the L30 protein can be engineered into phage genomic DNA and co-expressed with the phage coat protein. This should provide an excellent opportunity to explore the specific recognition principles between the L30 protein and RNA.

7.4.2. Crystallization of L30 protein-RNA complex with U1 snRNP fragment

One of the important features of the L30 protein is the regulation of splicing of the ribosomal protein transcript. This feature provides a rationale for the presence of introns in two-thirds of the ribosomal protein gene, whereas they are rare in other genes in yeast. The presence of an accumulated unspliced precursor molecule in the presence of L30 protein presents an opportunity for studying the splicing. More specifically, there seems to be a coexistence of L30 protein and U1 snRNP on the unspliced pre-mRNA transcript. Biochemistry studies are necessary to map out the minimal fragment of the tertiary complex. Furthermore, a revelation of the three component interactions in a crystal structure would present a satisfying explanation for

the presence of introns as a general mechanism for regulation of ribosomal protein production in

yeast.

Appendix A. Data Processing in NMRPipe

This appendix provides brief NMR data processing protocols using NMRPipe program (Delaglio *et al.*, 1995). Since data were recorded on two types of spectrometers (namely Varian and MIT Francis-Bitter Magnet Lab), different protocols were used to convert the data into NMRPipe readable format prior to the application of the window functions. These files are indicated as either "*var2pipe.com" for the Varian data or "*bin2pipebit.com" for the MITFBL data in the following protocols. Shown below are four protocols typical for processing (1) 2D Varian data, (2) 3D Varian data, (3) 2D MITFBL data, and (4) 3D MITFBL data. Detail parameters should consult the NMRPipe manual.

(1) **var2pipe.com**

```
#! /bin/csh

#var2pipe.com
#converting varian 2D data to NMRPipe format
#
var2pipe -in    fid                                    \
-xN       8192    -      yN       1024  \
-xT       4096           -yT      512   \
-xMODE    Complex        -yMODE   Complex\
-xSW      6600           -ySW     6600  \
-xOBS     599.944        -yOBS    599.944\
-xCAR     4.73    -      yCAR     4.73  \
-xLAB     F2              -yLAB    F1    \
-ndim     2              -aq2D    States \
#processing first and second dimensions
| NMRPipe -fn EM -lb -15.0                          \
| NMRPipe -fn GM -g2 16.0                           \
| NMRPipe -fn FT -verb                              \
| NMRPipe -fn PS -p0 1.2 -p1 0 -di                  \
| NMRPipe -fn POLY -auto -ord 1                     \
| NMRPipe -fn TP                                    \
| NMRPipe -fn EM -lb -10.0                          \
| NMRPipe -fn GM -g2 17.0                           \
| NMRPipe -fn ZF -size 1024                         \
| NMRPipe -fn FT -verb                              \
| NMRPipe -fn PS -p0 0 -p1 0 -di                    \
| NMRPipe -fn POLY -auto -ord 1                     \
```

```
        -ov -out data.ft2
```

(2) **bin2pipebit.com**

```
#! /bin/csh

#bin2pipebit.com
#converting Bitter Lab data to NMRPipe format
#
bin2pipe -in datar.dat   \
datai.dat  \
-xN          8192    -    yN          1024  \
-xT          4096         -yT         512    \
-xMODE       Complex       -yMODE      Complex\
-xSW         6002.4        -ySW        6002.4 \
-xOBS        501.85        -yOBS       501.85 \
-xCAR        4.91          -yCAR       4.91   \
-xLAB        H1            -yLAB       H1     \
-ndim        2            -aq2D       States  \
-bad 0.0 -out fid  -ov -verb -bo 1044 -bp 12 -ri
#processing first and second dimensions
NMRPipe -in fid                                  \
| NMRPipe -fn SOL                                \
| NMRPipe -fn CBF -last 10                       \
| NMRPipe -fn EM -lb -15.0                       \
| NMRPipe -fn GM -g2 15.0                        \
| NMRPipe -fn FT -verb                           \
| NMRPipe -fn REV                                \
| NMRPipe -fn PS -p0 -18.7 -p1 35.8 -di          \
| NMRPipe -fn POLY -auto -ord 1                  \
| NMRPipe -fn TP                                 \
| NMRPipe -fn EM -lb -15.0                       \
| NMRPipe -fn GM -g2 20.0                        \
| NMRPipe -fn ZF -auto                      \
| NMRPipe -fn FT -verb                           \
| NMRPipe -fn REV                                \
| NMRPipe -fn PS -p0 0 -p1 -9.4 -di              \
| NMRPipe -fn POLY -auto -ord 1                  \
        -ov -out data.ft2
```

(3) **3dvar2pipe.com**
```
#!/bin/csh
```

```
#3dvar2pipe.com
# Varian 3D States-Mode 750 3dwg Conversion:
#"-aqORD 1" selects for (3D d3,d2,phase,phase2) acquisition.

var2pipe -in fid \
-xN        1024       -yN       64         -zN       248        \
-xT        512        -yT       32         -zT       124        \
-xMODE     Complex    -yMODE    Complex    -zMODE    Complex    \
-xSW       9000       -ySW      2600       -zSW      9000       \
-xOBS      750.079    -yOBS     75.01      -zOBS     750.079    \
-xCAR      4.78       -yCAR     116.34     -zCAR     4.78       \
-xLAB      H1         -yLAB     N15        -zLAB     H1         \
-ndim3     -aq2D      States    -aqORD 1                        \
-out data%03d.fid -verb -ov

#processing first and second dimension
xyz2pipe -in data%02d.fid -x                        \
| NMRPipe -fn SOL                                   \
| NMRPipe -fn SP -off .45 -end 0.98 -pow 3 -c 1.0   \
| NMRPipe -fn FT -verb                              \
| NMRPipe -fn REV                                   \
| NMRPipe -fn PS -p0 108 -p1 0 -di                  \
| NMRPipe -fn EXT -x1 6.0ppm -xn 9.5ppm -sw         \
| NMRPipe -fn POLY -auto -ord 1                     \
| NMRPipe -fn TP                                    \
| NMRPipe -fn LP -after -pred 128                   \
| NMRPipe -fn SP -off 0.45 -end 0.98 -pow 4 -c 1.0 \
| NMRPipe -fn ZF -auto                                          \
| NMRPipe -fn FT -verb                              \
| NMRPipe -fn PS -p0 -40 -p1 0 -di                  \
| NMRPipe -fn REV                                   \
| NMRPipe -fn EXT -x1 -0.3ppm -xn 9.5ppm -sw        \
| NMRPipe -fn POLY -auto -ord 1                     \
| pipe2xyz -out data%2d.ft2 -y

#processing third dimension
xyz2pipe -in data%2d.ft2 -z              \
| NMRPipe -fn LPC                                   \
| NMRPipe -fn SP -off 0.5 -end 1.0 -pow 3 -c 1.0   \
| NMRPipe -fn FT -verb                             \
| NMRPipe -fn PS -p0 0 -p1 0 -di                   \
| pipe2xyz -out data%03d.ft3 -z
```

(4) 3dbin2pipe.com

```
#!/bin/csh
#
#3dbin2pipe.com
# shell to convert 3D bitter lab felix files to 3D NMRPipe files

set in = data
set out = data
set from = 1
set to = 32
set rr = rr
set ir = ir
set ri = ri
set ii = ii
@ totslice = $to * 2
if ( $totslice < 1000 ) then
        set dig = 3
endif
if ( $totslice < 100 ) then
        set dig = 2
endif
set zer = 0
echo "beginning conversion"
set n = $from
while ( $n <= $to )
@ num3di = $n * 2
@ num3dr = $num3di - 1
        if ( $num3dr < 10 ) then
                set num3dr = $zer$num3dr
        endif
        if ( $dig == 3 ) then
                if ( $num3dr < 100 ) then
                        set num3dr = $zer$num3dr
                endif
        endif
        if ( $num3di < 10 ) then
                set num3di = $zer$num3di
        endif
        if ( $dig == 3 ) then
                if ( $num3di < 100 ) then
                        set num3di = $zer$num3di
                endif
        endif
        echo  $in$rr$n.dat $in$ir$n.dat $out$num3dr.fid
```

```
bin2pipe -in $in$rr$n.dat $in$ir$n.dat \
-xN        1024        -yLAB      H1         -zLAB      15N      \
-xT        512         -yN        256        -zN        64       \
-xMODE     Complex     -yT        128        -zT        32       \
-xSW       6002.4      -yMODE     Complex    -zMODE     Complex\
-xOBS      501.86      -ySW       6002.4     -zSW       1600.0 \
-xCAR      4.91        -yOBS      501.86     -zOBS      50.856 \
-xLAB      H1          -yCAR      4.91       -zCAR      118      \
-ndim 2    -aq2D States                                         \
-bad 0.0 -verb -bo 1044 -bp 12 -ri                              \
-out $out$num3di.fid -fmt -ov


echo  $in$ri$n.dat $in$ii$n.dat $out$num3di.fid


bin2pipe -in $in$ri$n.dat $in$ii$n.dat \
-xN        1024        -yLAB      H1         -zLAB      15N      \
-xT        512         -yN        256        -zN        64       \
-xMODE     Complex     -yT        128        -zT        32       \
-xSW       6002.4      -yMODE     Complex    -zMODE     Complex\
-xOBS      501.86      -ySW       6002.4     -zSW       1600.0 \
-xCAR      4.91        -yOBS      501.86     -zOBS      50.856 \
-xLAB      H1          -yCAR      4.91       -zCAR      118      \
-ndim 2    -aq2D States                                         \
-bad 0.0 -verb -bo 1044 -bp 12 -ri                              \
-out $out$num3di.fid -fmt -ov
# reset ndim to 3
setfdata $out$num3dr.fid -ndim 3
setfdata $out$num3di.fid -ndim 3
@ n = $n + 1
end
exit
#processing first and second dimension
xyz2pipe -in data%02d.fid -x                        \
| NMRPipe -fn SOL                                   \
| NMRPipe -fn SP -off .45 -end 0.98 -pow 3 -c 1.0   \
| NMRPipe -fn FT -verb                              \
| NMRPipe -fn REV                                   \
| NMRPipe -fn PS -p0 108 -p1 0 -di                  \
| NMRPipe -fn EXT -x1 6.0ppm -xn 9.5ppm -sw         \
| NMRPipe -fn POLY -auto -ord 1                     \
| NMRPipe -fn TP                                    \
| NMRPipe -fn LP -after -pred 128                   \
| NMRPipe -fn SP -off 0.45 -end 0.98 -pow 4 -c 1.0  \
| NMRPipe -fn ZF -auto                              \
| NMRPipe -fn FT -verb                              \
```

```
| NMRPipe -fn PS -p0 -40 -p1 0 -di                    \
| NMRPipe -fn REV                                     \
| NMRPipe -fn EXT -x1 -0.3ppm -xn 9.5ppm -sw          \
| NMRPipe -fn POLY -auto -ord 1                       \
| pipe2xyz -out data%2d.ft2 -y

#processing third dimension
xyz2pipe -in data%2d.ft2 -z                  \
| NMRPipe -fn LPC                                     \
| NMRPipe -fn SP -off 0.5 -end 1.0 -pow 3 -c 1.0      \
| NMRPipe -fn FT -verb                                \
| NMRPipe -fn PS -p0 0 -p1 0 -di                      \
| pipe2xyz -out data%03d.ft3 -z
```

Appendix B. Modeling Protocol

This section is a reference to details of the modeling protocols used for calculating L30 protein-RNA complex structure in the X-PLOR program. The procedure used for generating the initial protein structures involves three protocols: randomhm.inp, dgsahm.inp and refinehm.inp, which are adopted from the X-PLOR tutorial section for generating protein structure. The procedure used for generating the initial RNA structures involves two protocols: rnainit.inp and rnarefine.inp, which are adopted from Brodsky's RNA modeling protocol. The complex structure are generated from docking of the initial protein structures to the initial RNA structures from a given distance and random orientation. The procedure involves three protocols: doc.inp, complexr.inp and complexr2.inp, which are based on the RNA protocols and refined during the modeling. The X-PLOR manual should be consulted for specific information on the parameters used in these protocols.

```
remarks nmr/randomhm.inp
remarks  The ultimate simulated annealing protocol for NMR structure
remarks  determination!
remarks  The starting structure for this protocol can be completely
remarks  arbitrary, such as random numbers.  Note: the resulting
remarks  structures need to be further processed by the dgsa.inp protocol.
remarks  Reference: Nilges, Clore, and Gronenborn 1988a.
remarks Author: Michael Nilges
remarks Modified from the random.inp for L30 protein calculation by HM
{=====>}
evaluate ($init_t = 1000 )     {* Initial simulated annealing temperature.*}
parameter                              {*Read the parameter file.*}
     {=====>}
     @TOPPAR:parallhdg.pro
end
{=====>}
structure @L30P.psf end          {*Read the structure file.*}
noe
{=====>}
     reset
     nres=6000         {*Estimate greater than the actual number of NOEs.*}
     class all
     {=====>}
     @L30P_noe.tbl                    {*Read NOE distance ranges.*}
end
{=====>}
@L30P_dih.tbl                    {*Read dihedral angle restraints.*}
noe                    {*Parameters for NOE effective energy term.*}
```

```
          ceiling=1000
          averaging  * R-6
          potential  * soft
          scale     * 1
          sqoffset   * 0.0
          sqconstant * 1.0
          sqexponent * 2
          soexponent * 1
          asymptote  * 2.0              {* Initial value - modified later. *}
          rswitch    * 1.0
end
set message=off end
{* ============================================= Generate random coordinates.*}
{====>}
evaluate ($end_count=20)          {* Number of structures. *}
evaluate ($count = 0)
set seed=FOOBAR end
while ($count < $end_count ) loop main
      evaluate ($count=$count+1)

                                                    {* Generate a starting structure. *}
      vector do (x = (random()-0.5)*20) (all)
      vector do (y = (random()-0.5)*20) (all)
      vector do (z = (random()-0.5)*20) (all)
      vector do (fbeta=10) (all)     {*Friction coefficient for MD heatbath.*}
      vector do (mass=100) (all)          {*Uniform heavy masses to speed*}
                                                    {*molecular dynamics. *}

      parameter nbonds
          atom cutnb 100 tolerance 45 repel=1.2
          rexp=2 irexp=2 rcon=1.0 nbxmod 4
      end end
      flags exclude * include bonds angle impr vdw noe cdih  harm end
      evaluate ($knoe = 0.5)
      evaluate ($kbon = 0.00005 )           {* Bonds.         *}
      evaluate ($kang = 0.00005 )           {* Angles.        *}
      evaluate ($kimp = 0.0 )               {* Impropers.     *}
      evaluate ($kvdw = 0.1)                {* Vdw.           *}
      constraints
          interaction (not name ca) (all)
          weights bond $kbon angl $kang impr $kimp vdw 0 elec 0 end
          interaction (name ca) (name ca)
          weights bond $kbon angl $kang impr $kimp vdw $kvdw end
      end
       {* ===================================== ===========High temperature dynamics. *}
      vector do (vx = maxwell($init_t)) (all)
      vector do (vy = maxwell($init_t)) (all)
      vector do (vz = maxwell($init_t)) (all)
      evaluate ($timestep = 0.04)
      evaluate ($nstep = 100)
      while ($kbon < 0.01) loop stage1
          evaluate ($kbon = min(0.25, $kbon * 1.25))
          evaluate ($kang = $kbon)
          evaluate ($kimp = 0)
          noe scale * $knoe end
          restraints dihed scale 0. end
```

```
        constraints
            interaction (not name ca) (all)
            weights bond $kbon angl $kang impr $kimp vdw 0 elec 0 end
            interaction (name ca) (name ca)
            weights bond $kbon angl $kang impr $kimp vdw $kvdw end
        end
        dynamics verlet
            nstep=$nstep  timestep=$timestep  iasvel=current
            tcoupling=true tbath=$init_t nprint=50 iprfrq=0
        end
end loop stage1
restraints dihed scale 0. end
noe scale * 5 end
parameter              {* Parameters for the repulsive energy term. *}
    nbonds                    .
        repel=0.9        {* Initial value for repel - modified later. *}
        nbxmod=-3          {* Initial value for nbxmod - modified later. *}
        wmin=0.01
        cutnb=4.5 ctonnb=2.99 ctofnb=3.
        tolerance=0.5
    end
end
constraints
    interaction (all) (all)
    weights bond 0.02 angl 0.02 impr 0 vdw 0.002 elec 0 end
end
dynamics verlet
    nstep=500  timestep=0.003  iasvel=maxwell
    firstt=1500
    tcoupling=true
    tbath=1500 nprint=50 iprfrq=0
end
constraints
    interaction (all) (all)
    weights bond 0.05 angl 0.05 impr 0 vdw 0.005 elec 0 end
end
dynamics verlet
    nstep=500  timestep=0.003  iasvel=current tcoupling=true
    tbath=1500 nprint=50 iprfrq=0
end
constraints
    interaction (all) (all)
    weights bond 0.1 angl 0.1 impr 0 vdw 0.01 elec 0 end
end
dynamics verlet
    nstep=500  timestep=0.003  iasvel=current tcoupling=true
    tbath=1500 nprint=50 iprfrq=0
end
    {* ============================================= Write out the final structure(s).*}
remarks ==================================================================
remarks         overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
{===>}                 {*Name(s) of the family of final structures.*}
evaluate ($filename="random_"+encode($count)+".pdb")
```

```
       write coordinates output =$filename end
end loop main
stop




remarks file nmr/dgsahm.inp -- Simulated annealing regularization
remarks                    and refinement for embedded distance
remarks                    geometry structures or substructures.
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger
remarks  modified from dgsa.inp for L30 calculation
{====>}
evaluate ($init_t = 2000)         {*Initial annealing temperature, in K.*}
{====>}
evaluate ($high_steps = 1000 )    {*Total number of steps at high temp.*}
{====>}
evaluate ($cool_steps = 1000 )    {*Total number of steps for cooling.*}
parameter                         {*Read the parameter file.*}
{====>}
       @TOPPAR:parallhdg.pro
end
{====>}
structure @L30P.psf end           {*Read the structure file.*}
noe
    {====>}
    reset
    nres=6000        {*Estimate greater than the actual number of NOEs.*}
    class all
    {====>}
    @L30P_noe.tbl                 {*Read NOE distance ranges.*}
end
{====>}
@L30P_dih.tbl              {*Read dihedral angle restraints.*}
vector do (fbeta=10) (all) {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (mass=100) (all)        {*Uniform heavy masses to speed MD.*}


noe                {*Parameters for NOE effective energy term.*}
    ceiling =1000
    averaging  * R-6
    potential  * square
    sqconstant * 1.
    sqexponent * 2
    scale    * 50.      {*Constant NOE scale throughout the protocol.*}
end
parameter                  {*Parameters for the repulsive energy term.*}
    nbonds
        repel=0.5              {*Initial value for repel--modified later.*}
        rexp=2 irexp=2 rcon=1.
        nbxmod=-2             {*Initial value for nbxmod--modified later.*}
        wmin=0.01
        cutnb=4.5 ctonnb=2.99 ctofnb=3.
        tolerance=0.5
    end
end
```

```
restraints dihedral  scale=5.              {*Initial weight--modified later.*}
end
{====>}
set seed=FOOBAR end
evaluate ($end_count=20)        {*Loop through a family of 50 structures.*}
evaluate ($count = 0)
while ($count < $end_count ) loop main
     evaluate ($count=$count+1)
     {====>}                    {*Filename(s) for embedded coordinates.*}
     evaluate ($filename="random_"+encode($count)+".pdb")
                                              {*Test for the correct enantiomer;       *}
     {====>}             {*if you want to bypass this test because the *}
                     {*substructures were tested previously, simply*}
                     {*remove the -1 from the next statement.    *}
     for $image in ( 1 -1 ) loop imag
          coor initialize end
          coor @@$filename
          vector do (x=x * $image) ( known )
          vector identity (store1) (not known)      {*Set store1 to unknowns.*}
          {* ==================================== Create local ideal geometry by template fitting;*}
          {*            this also takes care of unknown atoms.       *}
          set message=off echo=off end
          coor copy end        {*Store current coordinates in comparison set.*}
          {====>}              {*The user has to supply a template coordinate set.*}
          coor @@generate_template.pdb
          for $id in id ( tag ) loop fit          {*Loop over residue tags.*}
               coordinates                {*LSQ fitting using known coordinates.*}
               fit select = ( byresidue (id $id) and not store1 )
               end
                                                   {*Store fitted template coordinates for this residue.*}
               coor copy selection=( byresidue (id $id) ) end
          end loop fit
          coor swap end
          set message=on echo=on end
          {* ===================================== Minimization of bonds, VDWs, and NOEs.*}
          restraints dihedral  scale=5.  end
          parameter nbonds  nbxmod=-2 repel=0.5  end  end
          flags exclude * include bond vdw noe cdih end
          constraints interaction (all) (all) weights * 1.  vdw 20. end end
          minimize powell nstep=100 nprint=10 end
          {* ==================================================Include angles. *}
          flags include angl end
          minimize powell nstep=100 nprint=10 end
          {* ============================ Dynamics, slowly introducing chirality and planarity. *}
          flags include impr end
          evaluate ($nstep1 = int($high_steps/8))
          evaluate ($nstep2 = int($high_steps/2))
          constraints inter (all) (all) weights * 0.1 impr 0.05 vdw 20. End end
          dynamics  verlet
               nstep=$nstep1 time=0.003 iasvel=maxwell firstt=$init_t
               tcoup=true tbath=$init_t nprint=100 iprfrq=0
          end
          constraints inter (all) (all) weights * 0.2 impr 0.1  vdw 20. End end
          dynamics  verlet
```

```
        nstep=$nstep1 time=0.003 iasvel=current firstt=$init_t
        tcoup=true tbath=$init_t nprint=100 iprfrq=0
end
parameter nbonds repel=0.9  end  end
constraints inter (all) (all) weights * 0.2 impr 0.2 vdw 0.01 end end
dynamics  verlet
        nstep=$nstep1 time=0.003 iasvel=current firstt=$init_t
        tcoup=true tbath=$init_t nprint=100 iprfrq=0
end
parameter nbonds nbxmod=-3 end  end
constraints inter (all) (all) weights * 0.4 impr 0.4 vdw 0.003 end end
dynamics  verlet
        nstep=$nstep2 time=0.003 iasvel=current firstt=$init_t
        tcoup=true tbath=$init_t nprint=100 iprfrq=0
end
constraints inter (all) (all) weights * 1.0 impr 1.0 vdw 0.003 end end
dynamics  verlet
        nstep=$nstep1 time=0.003 iasvel=current  firstt=$init_t
        tcoup=true tbath=$init_t nprint=100 iprfrq=0
end
if ($image = 1) then
        vector do (store7=x) ( all )  {*Store first image in stores.*}
        vector do (store8=y) ( all )
        vector do (store9=z) ( all )
        vector do (store4=vx) ( all )
        vector do (store5=vy) ( all )
        vector do (store6=vz) ( all )
    end if
end loop imag
   {* ======================= ===================Establish the correct handedness of the structure.*}
energy end
evaluate ($e_minus=$ener)
coor copy end
vector do (x=store7) ( all )
vector do (y=store8) ( all )
vector do (z=store9) ( all )
energy end
evaluate ($e_plus=$ener)
if ( $e_plus > $e_minus ) then
    evaluate ($hand=-1 )
    coor swap end
    else
    evaluate ($hand= 1 )
    vector do (vx=store4) ( all )
    vector do (vy=store5) ( all )
    vector do (vz=store6) ( all )
end if
   {* ============================ ===============Increase VDW interaction and cool.*}
restraints dihedral  scale=200.  end
evaluate ($final_t = 100)    { K }
evaluate ($tempstep = 50)    { K }
evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
evaluate ($nstep = int($cool_steps/$ncycle))
evaluate ($ini_rad  = 0.9)       evaluate ($fin_rad  = 0.75)
```

```
evaluate ($ini_con= 0.003)    evaluate ($fin_con= 4.0)
evaluate ($bath = $init_t)
evaluate ($k_vdw = $ini_con)
evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
evaluate ($radius= $ini_rad)
evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
     evaluate ($i_cool=$i_cool+1)
     evaluate ($bath = $bath - $tempstep)
     evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
     evaluate ($radius=max($fin_rad,$radius*$radfact))
     parameter nbonds repel=$radius  end end
     constraints interaction (all) (all) weights * 1. vdw $k_vdw end end
     dynamics verlet
          nstep=$nstep time=0.005 iasvel=current firstt=$bath
          tcoup=true tbath=$bath nprint=$nstep iprfrq=0
     end
     {====>}                          {*Abort condition.*}
     evaluate ($critical=$temp/$bath)
     if ($critical > 10. ) then
          display ****&&&& rerun job with smaller timestep (i.e., 0.003)
          stop
     end if
end loop cool
     {* ==================================================Final minimization.*}
minimize powell nstep= 200 nprint=25 end
     {* ===================== ===================Analyze and write out the final structure(s).*}
print threshold=0.5 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks ================================================================
remarks         overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ================================================================
remarks         bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ================================================================
remarks         noe, cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ================================================================
remarks handedness: $hand, enantiomer discrimination ( $e_plus : $e_minus )
{====>}                    {*Name(s) of the family of final structures.*}
evaluate ($filename="dgsa_"+encode($count)+".pdb")
write coordinates output =$filename end
```

```
end loop main
stop
```

```
remarks file nmr/refinehm.inp -- Simulated annealing refinement
remarks                 for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger
remarks  adapted from refine.inp by hm for L30P 2/19/98
{====>}
evaluate ($init_t = 1000)        {*Initial annealing temperature, in K.*}
{====>}
evaluate ($cool_steps = 2000 )    {*Total number of steps during cooling.*}
parameter                         {*Read the parameter file.*}
    {====>}
    @TOPPAR:parallhdg.pro
end
{====>}
structure @L30P.psf end           {*The structure file.*}
noe
    {====>}
    reset
    nres=6000        {*Estimate greater than the actual number of NOEs.*}
    class all
    {====>}
    @L30P_noe.tbl                 {*Read NOE distance ranges.*}
    @L30P_hbond.tbl
end
{====>}
@L30P_dih.tbl                 {*Read dihedral angle restraints.*}
               {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)     {*Heavy masses to speed molecular dynamics.*}
noe                    {*Parameters for NOE effective energy term.*}
    ceiling=1000
    averaging  * R-6
    potential  * square
    sqconstant * 1.
    sqexponent * 2
    scale     * 50.        {*Constant NOE scale throughout the protocol.*}
end
parameter              {*Parameters for the repulsive energy term.*}
    nbonds
        repel=0.5              {*Initial value for repel--modified later.*}
        rexp=2 irexp=2 rcon=1.
        nbxmod=3
        wmin=0.01
        cutnb=4.5 ctonnb=2.99 ctofnb=3.
        tolerance=0.5
    end
end
restraints dihedral  scale=100  end
{====>}
evaluate ($end_count=20)       {*Loop through a family of 50 structures.*}
evaluate ($count = 0)
```

```
while ($count < $end_count ) loop main
    evaluate ($count=$count+1)
    {=====>}                   {*Filename(s) for embedded coordinates.*}
    evaluate ($filename="dgsa_"+encode($count)+".pdb")
    coor @@$filename
    flags exclude * include bond angl impr vdw noe cdih end
    vector do (vx=maxwell($init_t)) ( all )
    vector do (vy=maxwell($init_t)) ( all )
    vector do (vz=maxwell($init_t)) ( all )
    evaluate ($final_t = 100) { K }
    evaluate ($tempstep = 50) { K }
    evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
    evaluate ($nstep = int($cool_steps/$ncycle))
    evaluate ($ini_rad = 0.9)  evaluate ($fin_rad = 0.75)
    evaluate ($ini_con= 0.003) evaluate ($fin_con= 4.0)
    evaluate ($bath = $init_t)
    evaluate ($k_vdw = $ini_con)
    evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
    evaluate ($radius= $ini_rad)
    evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
    evaluate ($i_cool = 0)
    while ($i_cool < $ncycle) loop cool
        evaluate ($i_cool=$i_cool+1)
        evaluate ($bath = $bath - $tempstep)
        evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
        evaluate ($radius=max($fin_rad,$radius*$radfact))
        parameter nbonds repel=$radius  end end
        constraints interaction (all) (all) weights * 1. vdw $k_vdw end end
        dynamics  verlet
            nstep=$nstep time=0.005 iasvel=current firstt=$bath
            tcoup=true tbath=$bath nprint=$nstep iprfrq=0
        end
        {=====>}                          {*Abort condition.*}
        evaluate ($critical=$temp/$bath)
        if ($critical > 10. ) then
            display ****&&&& rerun job with smaller timestep (i.e., 0.003)
            stop
        end if
    end loop cool
    {* =============================================================== Final minimization.*}
    minimize powell nstep= 200 nprint=25 end
    {* =============================================================== Write out the final structure(s).*}
    print threshold=0.5 noe
    evaluate ($rms_noe=$result)
    evaluate ($violations_noe=$violations)
    print threshold=5. cdih
    evaluate ($rms_cdih=$result)
    evaluate ($violations_cdih=$violations)
    print thres=0.05 bonds
    evaluate ($rms_bonds=$result)
    print thres=5. angles
    evaluate ($rms_angles=$result)
    print thres=5. impropers
    evaluate ($rms_impropers=$result)
```

```
remarks ===============================================================
remarks          overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ===============================================================
remarks          bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ===============================================================
remarks          noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ===============================================================
{====>}                    {*Name(s) of the family of final structures.*}
evaluate ($filename="refineP_"+encode($count)+".pdb")
write coordinates output =$filename end
end loop main
stop




remarks nmr/rnainit.inp
remarks  The ultimate simulated annealing protocol for NMR structure
remarks  determination!
remarks  The starting structure for this protocol can be completely
remarks  arbitrary, such as random numbers. Note: the resulting
remarks  structures need to be further processed by the dgsa.inp protocol.
remarks  Reference: Nilges, Clore, and Gronenborn 1988a.
remarks Author: Michael Nilges
remarks adapted by jrw 12/95
remarks  Add dihedral restraint 12/28/97 by HM
remarks  An A form pdb is put in for caluculation. 2/23/98 by HM


parameter                        {*Read the parameter file.*}
     @TOPPAR:gammahmao.par       {*This file is in subdirectory*}
end
structure @L30N.psf end          {*Read the structure file.*}
noe
     reset
     nres=6000       {*Estimate greater than the actual number of NOEs.*}
     class all
     {====>
     @L30N_noe.tbl               {*Read NOE distance ranges.*}
end
noe                   {*Parameters for NOE effective energy term.*}
          ceiling=1000
          averaging  * R-6
          potential  * soft
          scale    *    1
          sqoffset  * 0.0
          sqconstant * 1.0
          sqexponent * 2
          soexponent * 1
          asymptote  * 2.0
          rswitch   * 1.0
end
{====>}
@L30N_dih.tbl
```

```
restraints dihedral scale=1.0 end
set message=off end
evaluate ($end_count=40)                    {* Number of structures. *}
evaluate ($init_t = 1000 )    {* Initial simulated annealing temperature.*}
evaluate ($count = 0)
set seed=FOOBAR end
while ($count < $end_count ) loop main
    evaluate ($count=$count+1)
    evaluate ($filename="L30N.pdb")  {*Filename(s) for SA calculation.*}
    coor @@$filename
    vector do (fbeta=10) (all)     {*Friction coefficient for MD heatbath.*}
     vector do (mass=100) (all)         {*Uniform heavy masses to speed*}
                                            {*molecular dynamics.*}
    parameter nbonds
        atom cutnb 45 tolerance 100 repel=1.2
        rexp=2 irexp=2 rcon=1.0 nbxmod 4 wmin=0.01
        end
    end
    flags exclude * include bonds angle impr vdw noe cdih  harm end
    evaluate ($knoe = 0.5)
    evaluate ($kbon = 0.01 )                 {* Bonds.        *}
    evaluate ($kang = 0.01 )                 {* Angles.       *}
    evaluate ($kimp = 0.0  )                 {* Impropers.    *}
    evaluate ($kvdw = 0.01)                  {* Vdw.          *}
    constraints
        interaction (all) (all)
        weights bond $kbon angl $kang impr $kimp vdw $kvdw end
    end
    minimize powell
        nsteps=100 drop=10 nprint=20
    end
    {* ===============================================High temperature dynamics.*}
    vector do (vx = maxwell($init_t)) (all)
    vector do (vy = maxwell($init_t)) (all)
    vector do (vz = maxwell($init_t)) (all)
    evaluate ($timestep = 0.003)
    evaluate ($nstep = 1000)
    while ($kbon < 0.01) loop stage1
        evaluate ($kbon = min(0.25, $kbon * 1.10))
        evaluate ($kang = $kbon)
        evaluate ($kimp = 0)
        noe scale * $knoe end
        restraints dihed scale 0.0 end
        constraints
            interaction (all) (all)
            weights bond $kbon angl $kang impr $kimp vdw $kvdw end
        end
        dynamics verlet
            nstep=$nstep  timestep=$timestep  iasvel=current
            tcoupling=true tbath=$init_t nprint=50 iprfrq=0
        end
    end loop stage1
    noe scale * 50 end
    parameter                      {* Parameters for the repulsive energy term. *}
```

```
    nbonds
        repel=0.9              {* Initial value for repel - modified later. *}
        nbxmod=-3              {* Initial value for nbxmod - modified later. *}
        wmin=0.01
        cutnb=4.5 ctonnb=2.99 ctofnb=3.
        tolerance=0.5
    end
end
evaluate ($kbon= 0.01)
evaluate ($kvdw = 0.01)
                                                                    {* initialize dynamics *}

constraints
    interaction (all) (all)
    weights bond $kbon angl $kbon impr $kbon vdw $kvdw elec 0 end
end
dynamics  verlet
    nstep=1000  timestep=0.003  iasvel=maxwell
    firstt=$init_t
    tcoupling=true
    tbath=$init_t nprint=50 iprfrq=0
end
                                                                    {* ramp up covalent terms *}

evaluate ($kvdw = .01)
while ($kbon < 1.0) loop rampcov
    evaluate ($kbon = $kbon*1.25)
    constraints
        interaction (all) (all)
        weights bond $kbon angl $kbon impr $kbon vdw $kvdw elec 0 end
    end
    dynamics  verlet
        nstep=1000  timestep=0.003  iasvel=current
        tcoupling=true
        tbath=$init_t nprint=50 iprfrq=0
    end
end loop rampcov
restraints dihed scale 200. end
flags exclude * include bonds angle impr vdw noe cdih dihe end
                                                                    {* ramp up vdw *}
evaluate ($kbon=1.0)
evaluate ($kvdw=0.01)
while ($kvdw < 1.0) loop rampvdw
    evaluate ($kvdw = $kvdw*1.5)
    constraints
        interaction (all) (all)
        weights bond $kbon angl $kbon impr $kbon vdw $kvdw elec 0 end
    end
    dynamics  verlet
        nstep=1000  timestep=0.003  iasvel=current
        tcoupling=true
        tbath=$init_t nprint=50 iprfrq=0
    end
end loop rampvdw
{* =================================================ut the final structure(s).*}
{===>}                  {*Name(s) of the family of final structures.*}
```

```
    evaluate ($filename="randomR_"+encode($count)+".pdb")
    write coordinates output =$filename end
end loop main
stop
```

```
remarks file nmr/rnarefine.inp       -- Gentle simulated annealing refinement
remarks                    for NMR structure determination
remarks based on pardi annealing protocol from Biochemistry on small loop.
remarks adapted by hm 3/4/98
{====>}
evaluate ($init_t = 1000)            {*SA temperature, in K.*}
{====>}
evaluate ($second_t = 600)
{====>}
evaluate ($third_t = 400)
{====>}
evaluate ($cool_steps =10000 )       {*Total number of SA steps.*}
parameter                      {*Read the parameter file.*}
{====>}
      @TOPPAR:gammahmao.par
end
{====>} structure @L30N.psf end      {*Read the structure file.*}
noe
     reset
     nres=3000         {*Estimate greater than the actual number of NOEs.*}
     class all
     {====>}
     @L30N_noe.tbl                   {*Read NOE distance ranges.*}
end
noe                    {*Parameters for NOE effective energy term.*}
     ceiling=1000
     averaging  * R-6
     potential  * square
     scale    * 50
     sqoffset  * 0.0
     sqconstant * 1.
     sqexponent * 2
     soexponent * 1
     asymptote  * 2.0              {* Initial value - modified later. *}
     rswitch   * 1.0
end
parameter
     nbonds
     repel=0.9
     rexp=2 irexp=2 rcon=1.
     nbxmod=-5
     wmin=0.01
     cutnb=4.5 ctonnb=2.99 ctofnb=3.
     tolerance=1
     end
end
{====>}
@L30N_dih.tbl                   {*Read dihedral angle restraints.*}
```

restraints dihedral scale=50. end   {* scale above 100 is bad base on nt. tests *}
{====>}
set seed=FOOBAR end
evaluate ($end_count=40)   {*Loop through a family of 7 structures.*}
evaluate ($count = 0)
while ($count < $end_count) loop main
     evaluate ($count=$count+1)
     {====>}   {*Filename(s) for embedded coordinates.*}
     evaluate ($filename="randomR_"+encode($count)+".pdb")
     coor @@$filename
     flags exclude * include bond angl impr vdw noe cdih end
     {* in nucl. tests impr too high led to collapse of bases *}
     constraints
          interaction (all) (all)
          weights impr 0.5 end
     end
     {* === ==========Intial minimization.*}
     minimize powell nstep= 100 nprint=25 end
     {* == Constant temperature SA.*}
     vector do (fbeta=100.) (all)   {* Coupling to heat bath. *}
     vector do (mass=100.) (all)   {* Set mass to heavy. *}
     dynamics verlet
          nstep=$cool_steps timestep=0.00075
          iasvel=maxwell firsttemperature=$init_t
          tbath=$init_t
          tcoup=true
          nprint=250 iprfrq=2500
     end
     {* === 700 step minimization.*}
     minimize powell nstep= 700 nprint=25 end
     {* === 600 K dynamics *}
     dynamics verlet
          nstep=$cool_steps timestep=0.00075
          iasvel=maxwell firsttemperature=$second_t
          tbath=$second_t
          tcoup=true
          nprint=250 iprfrq=2500
     end
     {* === ========700 step minimization.*}
     minimize powell nstep= 700 nprint=25 end
     {* === ============400 K dynamics *}
     dynamics verlet
          nstep=$cool_steps timestep=0.00075
          iasvel=maxwell firsttemperature=$third_t
          tbath=$third_t
          tcoup=true
          nprint=250 iprfrq=2500
     end
     {* === ============Final minimization.*}
     minimize powell nstep= 1000 nprint=25 end
     {* === ============Write out the final structure(s).*}
     print threshold=0.5 noe
     evaluate ($rms_noe=$result)
     evaluate ($violations_noe=$violations)

```
          print threshold=5. cdih
          evaluate ($rms_cdih=$result)
          evaluate ($violations_cdih=$violations)
          print thres=0.05 bonds
          evaluate ($rms_bonds=$result)
          print thres=5. angles
          evaluate ($rms_angles=$result)
          print thres=5. impropers
          evaluate ($rms_impropers=$result)
          print thres=5. dihedrals
          evaluate ($rms_dihedrals=$result)
          remarks ===============================================================
          remarks          overall,bonds,angles,improper,vdw,noe,cdih
          remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
          remarks ===============================================================
          remarks          bonds,angles,impropers,noe,cdih
          remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
          remarks ===============================================================
          remarks          noe, cdih
          remarks violations.: $violations_noe, $violations_cdih
          remarks ===============================================================
          {====>}                    {*Name(s) of the family of final structures.*}
          evaluate ($filename="refineR_"+encode($count)+".pdb")
          write coordinates output =$filename end
   end loop main
   stop




   remarks file nmr/doc.inp   -- Docking of protein to RNA
   remarks  including rotating protein and send away at random angle.
   {====>}
   evaluate ($init_t = 1000)        {*Initial annealing temperature, in K.*}
   {====>}     {*Total number of steps during constanttemperature SA.*}
   evaluate ($ctemp_steps = 50000 )
   {====>}
   parameter                        {*Read the parameter file.*}
        @TOPPAR:gammahmao.par              {*This file is in subdirectory*}
   end
   parameter                        {*Read the parameter file.*}
   {====>}
        @TOPPAR:parallhdg.pro
   end
   {====>}
   structure @L30C.psf end          {*The structure file.*}
   noe
   {====>}
        reset
        nres=6000          {*Estimate greater than the actual number of NOEs.*}
        class rna  @L30N_noe.tbl
        class pro  @L30P_noe.tbl
                   @L30P_hbond.tbl
        class comp  @L30NP_noe.tbl
   end
   {====>}                           {*Read dihedral angle restraints.*}
```

```
@L30P_dih.tbl
@L30N_dih.tbl
                        {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)     {*Heavy masses to speed molecular dynamics.*}
noe                    {*Parameters for NOE effective energy term.*}
    ceiling=100
    averaging  *  R-6
    potential  * square
    sqoffset  * 0.0
    sqconstant * 1.
    sqexponent * 2
    soexponent * 1
    asymptote  * 2.0            {* Initial value - modified later. *}
    rswitch    * 1.0
end
parameter
    nbonds
    repel=0.9
    rexp=2 irexp=2 rcon=1.
    nbxmod=-2      {* excludes nonbonded interactions between bonded atoms *}
    wmin=0.01
    cutnb=4.5 ctonnb=2.99 ctofnb=3.
    tolerance=10
    end
end
restraints dihed scale 10. end
{====>}
set seed=FOOBAR end
evaluate ($end_count=20)        {*Loop through a family of 20 structures.*}
evaluate ($count = 0)
while ($count < $end_count ) loop main
    evaluate ($count=$count+1)
    {====>}                          {* protein structure *}
    evaluate ($filename="refineP_" + encode($count)+".pdb")
    coor @@$filename
    {* =============================================== rotate protein coordinates *}
    coordinates copy end
    evaluate ($t1 = random()*180)
    evaluate ($t2 = random()*360)
    evaluate ($t3 = random()*360)
    evaluate ($m11=cos($t1)*cos($t3)-sin($t1)*cos($t2)*sin($t3))
    evaluate ($m12=sin($t1)*cos($t3)+cos($t1)*cos($t2)*sin($t3))
    evaluate ($m13=sin($t2)*sin($t3))
    evaluate ($m21=-sin($t1)*cos($t2)*cos($t3)-cos($t1)*sin($t3))
    evaluate ($m22=-sin($t1)*sin($t3)+cos($t1)*cos($t2)*cos($t3))
    evaluate ($m23=sin($t2)*cos($t3))
    evaluate ($m31=sin($t1)*sin($t2))
    evaluate ($m32=-cos($t1)*sin($t2))
    evaluate ($m33=cos($t2))
    vector do (x=xcomp*$m11+ycomp*$m12+zcomp*$m13) (all)
    vector do (y=xcomp*$m21+ycomp*$m22+zcomp*$m23) (all)
    vector do (z=xcomp*$m31+ycomp*$m32+zcomp*$m33) (all)
                            {* translate protein coordinates was 70, too short. *}
```

```
evaluate ($tdist=100.0)
evaluate ($sx=random()-0.5)
evaluate ($sy=random()-0.5)
evaluate ($sz=random()-0.5)
evaluate ($len=sqrt($sx*$sx+$sy*$sy+$sz*$sz))
evaluate ($tx=$tdist*$sx/$len)
evaluate ($ty=$tdist*$sy/$len)
evaluate ($tz=$tdist*$sz/$len)
vector do (x=x+$tx) (all)
vector do (y=y+$ty) (all)
vector do (z=z+$tz) (all)
{====>}                             {* RNA structure *}
evaluate ($filename="refineR_" + encode($count)+".pdb")
coor @@$filename
flags exclude * include bond angl impr vdw noe cdih dihe end
restraints plane
    group
        selection =(
                (not ( name P   or name O1P or name O2P or name O5' or name H5T or
                    name H1' or name H2' or name H3' or name H4' or name H5' or
                    name O2' or name HO2' or name H5" or
                    name O3' {or name C1'} or name C2' or name C3' or name C4' or
                    name C5' or name O4' or name H3T) and residue 207)
                or (not ( name P   or name O1P or name O2P or name O5' or name H5T or
                    name H1' or name H2' or name H3' or name H4' or name H5' or
                    name O2' or name HO2' or name H5" or
                    name O3' {or name C1'} or name C2' or name C3' or name C4' or
                    name C5' or name O4' or name H3T) and residue 224)
        )
        weight=1.0
    end
end
flags include plan end        {* The planarity energy term needs to be turned on *}
constraints
    interaction (all) (all)
    weights * 1 impr 0.5 end
end
evaluate ($totsteps = 0)
{* ===================================================== ========Ramping up NOE during docking*}
vector do ( fbeta=100. ) ( all )        {* Coupling to heat bath. *}
vector do ( mass=100.  ) ( all )        {* Coupling to heat bath. *}
noe scale pro 50 end
noe scale rna 50 end
evaluate ($knoe = 0.001)
While ( $knoe < 50 ) loop noeramp
    noe scale comp $knoe end
    evaluate ($knoe = $knoe*1.5)
    dynamics  verlet
        nstep=2000 timestep=0.003
        iasvel=maxwell firsttemperature=$init_t
        tbath=$init_t
        tcoup=true
        nprint=250 iprfrq=2500
        end
```

```
end loop noeramp
{* ======================================================== ===========Final minimization.*}
minimize powell nstep= 200 nprint=25 end
{* ==================================================Write out the final structure(s).*}
print threshold=0.5 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks ======================================================
remarks         overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ====================================================== ===========
remarks         bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ======================================================
remarks          noe, cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ======================================================
{====>}                  {*Name(s) of the family of final structures.*}
evaluate ($filename="doc_"+encode($count)+".pdb")
write coordinates output =$filename end
end loop main
stop
```

```
remarks file nmr/complexr.inp        -- Gentle simulated annealing refinement
remarks                      for NMR structure determination
remarks based on pardi annealing protocol from Biochemistry on small loop.
remarks complex first refinement  hm 3/4/98
{====>}
evaluate ($init_t = 1000)                {*SA temperature, in K.*}
{====>}
evaluate ($second_t = 600)
{====>}
evaluate ($third_t = 400)
{====>}
evaluate ($cool_steps =10000 )           {*Total number of SA steps.*}
parameter
    @TOPPAR:gammahmao.par                {*This file is in subdirectory*}
end

parameter                        {*Read the parameter file.*}
    {====>}
    @TOPPAR:parallhdg.pro
end
{====>} structure @L30C.psf end           {*The structure file.*}
```

```
noe
    reset
    nres=6000         {*Estimate greater than the actual number of NOEs.*}
    class rna  @L30N_noe.tbl
    class pro  @L30P_noe.tbl
               @L30P_hbond.tbl
    class comp @L30NP_noe.tbl
end
{=====>}                      {*Read dihedral angle restraints.*}
@L30P_dih.tbl
@L30N_dih.tbl
noe                  {*Parameters for NOE effective energy term.*}
    ceiling=1000
    averaging  * R-6
    potential  * square
    scale      * 50
    sqoffset   * 0.0
    sqconstant * 1.
    sqexponent * 2
    soexponent * 1
    asymptote  * 2.0         {* Initial value - modified later. *}
    rswitch    * 1.0
end
parameter
    nbonds
    repel=0.9
    rexp=2 irexp=2 rcon=1.
    nbxmod=-5
    wmin=0.01
    cutnb=4.5 ctonnb=2.99 ctofnb=3.
    tolerance=1
    end
end
restraints dihedral scale=10. end    {* scale above 100 is bad base on nt. tests *}
{=====>}
set seed=FOOBAR end
evaluate ($end_count=20)        {*Loop through a family of 20 structures.*}
evaluate ($count = 0)
while ($count < $end_count ) loop main
    evaluate ($count=$count+1)
    {=====>}                    {*Filename(s) for embedded coordinates.*}
    evaluate ($filename="doc_"+encode($count)+".pdb")
    coor @@$filename
    flags exclude * include bond angl impr vdw noe cdih  end
    {* in nucl. tests impr too high led to collapse of bases *}
    constraints
        interaction (all) (all)
        weights impr 0.5 end
    end
    {* ================================================================Intial minimization.*}
    minimize powell nstep= 100 nprint=25 end
    {* ================================================ ==========Constant temperature SA.*}
    vector do ( fbeta=100. ) ( all )        {* Coupling to heat bath. *}
    vector do ( mass=100. ) ( all )         {* Set mass to heavy. *}
```

```
dynamics verlet
    nstep=$cool_steps timestep=0.00075
    iasvel=maxwell firsttemperature=$init_t
    tbath=$init_t
    tcoup=true
    nprint=250 iprfrq=2500
end
```

{* == 700 step minimization.*}
minimize powell nstep= 700 nprint=25 end
{* ==600 K dynamics *}

```
dynamics verlet
    nstep=$cool_steps timestep=0.00075
    iasvel=maxwell firsttemperature=$second_t
    tbath=$second_t
    tcoup=true
    nprint=250 iprfrq=2500
end
```
{* ==700 step minimization.*}
minimize powell nstep= 700 nprint=25 end
{* == 400 K dynamics *}
```
dynamics verlet
    nstep=$cool_steps timestep=0.00075
    iasvel=maxwell firsttemperature=$third_t
    tbath=$third_t
    tcoup=true
    nprint=250 iprfrq=2500
end
```
{* ==Final minimization.*}
minimize powell nstep= 1000 nprint=25 end
{* ==Write out the final structure(s).*}
print threshold=0.5 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
print thres=5. dihedrals
evaluate ($rms_dihedrals=$result)
remarks ==
remarks   overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ==
remarks   bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ==
remarks   noe, cdih
```

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```
      remarks violations.: $violations_noe, $violations_cdih
      remarks ====================================================================
      {====>}                 {*Name(s) of the family of final structures.*}
      evaluate ($filename="refine_"+encode($count)+".pdb")
      write coordinates output =$filename end
end loop main
stop



remarks file nmr/complexr2.inp     -- Gentle simulated annealing refinement
remarks                       for NMR structure determination
remarks based on pardi annealing protocol from Biochemistry on small loop.
remarks complex second refinement adapted by hm 3/4/98
{====>}
evaluate ($init_t = 1000)              {*SA temperature, in K.*}
{====>}
evaluate ($final_t = 10)
{====>}
evaluate ($cool_steps =10000 )         {*Total number of SA steps.*}
{====>}
parameter                    {*Read the parameter file.*}
      @TOPPAR:gammahmao.par            {*This file is in subdirectory*}
end
parameter                    {*Read the parameter file.*}
      @TOPPAR:parallhdg.pro
end
{====>} structure @L30C.psf end        {*The structure file.*}
noe
{====>}
      reset
      nres=6000       {*Estimate greater than the actual number of NOEs.*}
      class rna  @L30N_noe.tbl
      class pro  @L30P_noe.tbl
               @L30P_hbond.tbl
      class comp @L30NP_noe.tbl
end
{====>}                       {*Read dihedral angle restraints.*}
@L30P_dih.tbl
@L30N_dih.tbl
noe                  {*Parameters for NOE effective energy term.*}
      ceiling=1000
      averaging  * R-6
      potential  * square
      scale     * 50
      sqoffset  * 0.0
      sqconstant * 1.
      sqexponent * 2
      soexponent * 1
      asymptote  * 2.0          {* Initial value - modified later. *}
      rswitch    * 1.0
end
parameter
      nbonds
         repel=0.9
```

```
                rexp=2 irexp=2 rcon=1.
                nbxmod=-5
                wmin=0.01
                cutnb=4.5 ctonnb=2.99 ctofnb=3.
                tolerance=1
        end
end
restraints dihedral scale=10. end  {* scale above 100 is bad base on nt. tests *}
{====>}
set seed=FOOBAR end
evaluate ($end_count=20)        {*Loop through a family of 7 structures.*}
evaluate ($count = 0)
while ($count < $end_count ) loop main
        evaluate ($count=$count+1)
        {====>}                 {*Filename(s) for embedded coordinates.*}
        evaluate ($filename="refine_"+encode($count)+".pdb")
        coor @@$filename
        flags exclude * include bond angl impr vdw noe cdih  end
        restraints plane
        group
            selection =(
                (not ( name P   or name O1P or name O2P or name O5' or name H5T or
                    name H1' or name H2' or name H3' or name H4' or name H5' or
                    name O2' or name HO2' or name H5" or
                    name O3' {or name C1'} or name C2' or name C3' or name C4' or
                    name C5' or name O4' or name H3T) and residue 207)
                or (not ( name P   or name O1P or name O2P or name O5' or name H5T or
                    name H1' or name H2' or name H3' or name H4' or name H5' or
                    name O2' or name HO2' or name H5" or
                    name O3' {or name C1'} or name C2' or name C3' or name C4' or
                    name C5' or name O4' or name H3T) and residue 224)
            )
            weight=1.0
            end
        end
        flags include plan end      {* The planarity energy term needs to be turned on *}
        {* in nucl. tests impr too high led to collapse of bases *}
        constraints
            interaction (all) (all)
            weights impr 0.5 end
        end
        {* ============================================== Intial minimization.*}
        minimize powell nstep= 200 nprint=25 end
        {* ============================================= Constant temperature SA.*}
        vector do ( fbeta=100. ) ( all )        {* Coupling to heat bath. *}
        vector do ( mass=100. ) ( all )         {* Set mass to heavy. *}
        evaluate ($final_t = 100)    { K }
        evaluate ($tempstep = 10)    { K }
        evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
        evaluate ($nstep = int($cool_steps/$ncycle))
        evaluate ($ini_rad  = 0.9)  evaluate ($fin_rad  = 0.75)
        evaluate ($ini_con= 0.003) evaluate ($fin_con= 4.0)
        evaluate ($bath  = $init_t)
        evaluate ($k_vdw = $ini_con)
```

```
evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
evaluate ($radius=   $ini_rad)
evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
     evaluate ($i_cool=$i_cool+1)
     evaluate ($bath  = $bath - $tempstep)
     evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
     evaluate ($radius=max($fin_rad,$radius*$radfact))
     parameter nbonds repel=$radiusend end
     constraints interaction (all) (all) weights * 1. impr 0.5 vdw $k_vdw end end
     dynamics verlet
          nstep=$nstep time=0.00075 iasvel=maxwell firstt=$bath
          tcoup=true tbath=$bath nprint=$nstep iprfrq=0
     end
     {* ================================================================700 step minimization.*}
     minimize powell nstep= 100 nprint=25 end
     {====>}                         {*Abort condition.*}
     evaluate ($critical=$temp/$bath)
     if ($critical > 10. ) then
          display ****&&&& rerun job with smaller timestep (i.e., 0.003)
          stop
     end if
end loop cool
{* ==============================================================Final minimization.*}
minimize powell nstep= 1000 nprint=25 end
{* =========================================== ================Write out the final structure(s).*}
print threshold=0.5 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
print thres=5. dihedrals
evaluate ($rms_dihedrals=$result)
remarks =====================================================================
remarks         overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks =====================================================================
remarks         bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks =====================================================================
remarks          noe, cdih
remarks violations.: $violations_noe, $violations_cdih
remarks =====================================================================
{====>}                    {*Name(s) of the family of final structures.*}
evaluate ($filename="refine2_"+encode($count)+".pdb")
write coordinates output =$filename end
```

```
end loop main
stop
```

Appendix C. Restraints

Table C. Molecular modeling restraint files used in X-PLOR calculation (*.tbl). There are six "*.tbl" files in this table, including four distance restraints: L30P_noe.tbl, L30P_hbond.tbl, L30N_noe.tbl and L30NP_noe.tbl, and two dihedral restraints: L30P_dih.tbl and L30N_dih.tbl. The columns for distance restraints are as follows: (1) "assign", (2) "(resid", (3) residue #, (4) "and", (5) "name", (6) atom name ")", (7) "(resid", (8) residue #, (9) "and", (10) "name", (11) atom name ")",(12) distance, (13) d_{minus}, and (14) d_{plus} . The columns for dihedral angle restraints are similar, except for the following ones: (12) energy constant, (13) dihedral angle, (14) range around the restrained angle, and (15) exponent of the restraining function. Restraints are divided into various classes of restraints for clarity of reading. The different sections are headed by comments "!". **Note that the numbering of the RNA in the restraint file is different than the main body of the text.** The RNA is numbered as 201-233.

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!Protein intra- and inter- residue restraints, L30P_noe.tbl ,7/10/98
set echo = off end
set message = off end
!intra- ressidue restraints
!ALA
assign  (resid  2   and   name   HA  )  (resid  2   and   name   HB*  )  2.4   0.6   0.6
!PRO
assign  (resid  3   and   name   HB1 )  (resid  3   and   name   HA   )  2.4   0.6   0.6
assign  (resid  3   and   name   HB2 )  (resid  3   and   name   HA   )  2.4   0.6   0.6
assign  (resid  3   and   name   HD* )  (resid  3   and   name   HB1  )  3.9   2.1   2.1
assign  (resid  3   and   name   HD* )  (resid  3   and   name   HB2  )  3.9   2.1   2.1
assign  (resid  3   and   name   HG* )  (resid  3   and   name   HB1  )  2.4   0.6   0.6
assign  (resid  3   and   name   HG* )  (resid  3   and   name   HB2  )  2.4   0.6   0.6
assign  (resid  3   and   name   HG* )  (resid  3   and   name   HD*  )  2.4   0.6   0.6
!VAL
assign  (resid  4   and   name   HB  )  (resid  4   and   name   HA   )  2.9   1.1   1.1
assign  (resid  4   and   name   HG1*)  (resid  4   and   name   HA   )  2.4   0.6   0.6
assign  (resid  4   and   name   HG2*)  (resid  4   and   name   HA   )  2.4   0.6   0.6
assign  (resid  4   and   name   HG1*)  (resid  4   and   name   HB   )  2.9   1.1   1.1
assign  (resid  4   and   name   HG2*)  (resid  4   and   name   HB   )  2.9   1.1   1.1
assign  (resid  4   and   name   HN  )  (resid  4   and   name   HA   )  2.4   0.6   0.6
assign  (resid  4   and   name   HN  )  (resid  4   and   name   HB   )  2.4   0.6   0.6
assign  (resid  4   and   name   HN  )  (resid  4   and   name   HG2* )  2.9   1.1   1.1
assign  (resid  4   and   name   HN  )  (resid  4   and   name   HG1* )  3.4   1.6   1.6
!LYS
assign  (resid  5   and   name   HB1 )  (resid  5   and   name   HA   )  2.4   0.6   0.6
assign  (resid  5   and   name   HB2 )  (resid  5   and   name   HA   )  2.4   0.6   0.6
assign  (resid  5   and   name   HE* )  (resid  5   and   name   HA   )  3.4   1.6   1.6
assign  (resid  5   and   name   HE* )  (resid  5   and   name   HB*  )  3.4   1.6   1.6
assign  (resid  5   and   name   HE* )  (resid  5   and   name   HN   )  3.9   2.1   2.1
assign  (resid  5   and   name   HG* )  (resid  5   and   name   HB*  )  2.4   0.6   0.6
assign  (resid  5   and   name   HN  )  (resid  5   and   name   HA   )  2.9   1.1   1.1
assign  (resid  5   and   name   HN  )  (resid  5   and   name   HB1  )  2.4   0.6   0.6
assign  (resid  5   and   name   HN  )  (resid  5   and   name   HB2  )  2.9   1.1   1.1
assign  (resid  5   and   name   HN  )  (resid  5   and   name   HD*  )  3.9   2.1   2.1
assign  (resid  5   and   name   HN  )  (resid  5   and   name   HG*  )  3.4   1.6   1.6
!SER
assign  (resid  6   and   name   HB* )  (resid  6   and   name   HA   )  2.4   0.6   0.6
assign  (resid  6   and   name   HN  )  (resid  6   and   name   HA   )  2.4   0.6   0.6
assign  (resid  6   and   name   HN  )  (resid  6   and   name   HB*  )  2.9   1.1   1.1
!GLN
assign  (resid  7   and   name   HB1 )  (resid  7   and   name   HA   )  2.4   0.6   0.6
assign  (resid  7   and   name   HB2 )  (resid  7   and   name   HA   )  2.4   0.6   0.6
assign  (resid  7   and   name   HE2*)  (resid  7   and   name   HA   )  3.4   1.6   1.6
assign  (resid  7   and   name   HE2*)  (resid  7   and   name   HB*  )  3.9   2.1   2.1
assign  (resid  7   and   name   HG* )  (resid  7   and   name   HA   )  3.4   1.6   1.6
```

assign	(resid	7	and	name	HG*)	(resid	7	and	name	HB1)	2.4	0.6	0.6
assign	(resid	7	and	name	HG*)	(resid	7	and	name	HB2)	2.4	0.6	0.6
assign	(resid	7	and	name	HG*)	(resid	7	and	name	HE2*)	2.9	1.1	1.1
assign	(resid	7	and	name	HN)	(resid	7	and	name	HA)	2.4	0.6	0.6
assign	(resid	7	and	name	HN)	(resid	7	and	name	HB1)	2.9	1.1	1.1
assign	(resid	7	and	name	HN)	(resid	7	and	name	HB2)	3.4	1.6	1.6
assign	(resid	7	and	name	HN)	(resid	7	and	name	HE2*)	3.9	2.1	2.1
assign	(resid	7	and	name	HN)	(resid	7	and	name	HG*)	3.4	1.6	1.6

!GLU

assign	(resid	8	and	name	HB*)	(resid	8	and	name	HA)	2.4	0.6	0.6
assign	(resid	8	and	name	HG*)	(resid	8	and	name	HA)	2.9	1.1	1.1
assign	(resid	8	and	name	HG*)	(resid	8	and	name	HB*)	2.4	0.6	0.6
assign	(resid	8	and	name	HN)	(resid	8	and	name	HA)	2.4	0.6	0.6
assign	(resid	8	and	name	HN)	(resid	8	and	name	HB*)	2.9	1.1	1.1
assign	(resid	8	and	name	HN)	(resid	8	and	name	HG*)	2.9	1.1	1.1

!SER

assign	(resid	9	and	name	HB1)	(resid	9	and	name	HA)	2.9	1.1	1.1
assign	(resid	9	and	name	HB2)	(resid	9	and	name	HA)	2.4	0.6	0.6
assign	(resid	9	and	name	HN)	(resid	9	and	name	HA)	2.9	1.1	1.1
assign	(resid	9	and	name	HN)	(resid	9	and	name	HB1)	2.4	0.6	0.6
assign	(resid	9	and	name	HN)	(resid	9	and	name	HB2)	2.4	0.6	0.6

!ILE

assign	(resid	10	and	name	HB)	(resid	10	and	name	HA)	2.9	1.1	1.1
assign	(resid	10	and	name	HD1*)	(resid	10	and	name	HA)	3.4	1.6	1.6
assign	(resid	10	and	name	HD1*)	(resid	10	and	name	HB)	2.9	1.1	1.1
assign	(resid	10	and	name	HG1*)	(resid	10	and	name	HA)	2.9	1.1	1.1
assign	(resid	10	and	name	HG1*)	(resid	10	and	name	HB)	2.9	1.1	1.1
assign	(resid	10	and	name	HG1*)	(resid	10	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	10	and	name	HG2*)	(resid	10	and	name	HA)	3.4	1.6	1.6
assign	(resid	10	and	name	HG2*)	(resid	10	and	name	HB)	2.9	1.1	1.1
assign	(resid	10	and	name	HG2*)	(resid	10	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	10	and	name	HG2*)	(resid	10	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	10	and	name	HN)	(resid	10	and	name	HA)	2.9	1.1	1.1
assign	(resid	10	and	name	HN)	(resid	10	and	name	HB)	2.4	0.6	0.6
assign	(resid	10	and	name	HN)	(resid	10	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	10	and	name	HN)	(resid	10	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	10	and	name	HN)	(resid	10	and	name	HG2*)	2.9	1.1	1.1

!ASP

assign	(resid	11	and	name	HB*)	(resid	11	and	name	HA)	2.4	0.6	0.6
assign	(resid	11	and	name	HD2*)	(resid	11	and	name	HA)	3.4	1.6	1.6
assign	(resid	11	and	name	HD2*)	(resid	11	and	name	HB*)	2.4	0.6	0.6
assign	(resid	11	and	name	HN)	(resid	11	and	name	HA)	2.4	0.6	0.6
assign	(resid	11	and	name	HN)	(resid	11	and	name	HB*)	2.4	0.6	0.6
assign	(resid	11	and	name	HN)	(resid	11	and	name	HD2*)	3.9	2.1	2.1

!GLN

assign	(resid	12	and	name	HB2)	(resid	12	and	name	HA)	2.4	0.6	0.6
assign	(resid	12	and	name	HB1)	(resid	12	and	name	HA)	2.9	1.1	1.1
assign	(resid	12	and	name	HE2*)	(resid	12	and	name	HA)	3.9	2.1	2.1
assign	(resid	12	and	name	HE2*)	(resid	12	and	name	HB1)	3.9	2.1	2.1
assign	(resid	12	and	name	HE2*)	(resid	12	and	name	HB2)	3.4	1.6	1.6
assign	(resid	12	and	name	HG*)	(resid	12	and	name	HA)	2.9	1.1	1.1
assign	(resid	12	and	name	HG*)	(resid	12	and	name	HB2)	2.4	0.6	0.6
assign	(resid	12	and	name	HG*)	(resid	12	and	name	HE2*)	2.4	0.6	0.6
assign	(resid	12	and	name	HN)	(resid	12	and	name	HA)	2.4	0.6	0.6
assign	(resid	12	and	name	HN)	(resid	12	and	name	HB1)	2.9	1.1	1.1
assign	(resid	12	and	name	HN)	(resid	12	and	name	HB2)	2.4	0.6	0.6
assign	(resid	12	and	name	HN)	(resid	12	and	name	HG*)	3.4	1.6	1.6
assign	(resid	12	and	name	HN)	(resid	12	and	name	HE2*)	3.9	2.1	2.1

!LYS

assign	(resid	13	and	name	HA)	(resid	13	and	name	HD*)	3.9	2.1	2.1
assign	(resid	13	and	name	HB*)	(resid	13	and	name	HA)	2.9	1.1	1.1
assign	(resid	13	and	name	HB*)	(resid	13	and	name	HE*)	3.4	1.6	1.6
assign	(resid	13	and	name	HG*)	(resid	13	and	name	HA)	3.4	1.6	1.6
assign	(resid	13	and	name	HG*)	(resid	13	and	name	HE*)	2.9	1.1	1.1
assign	(resid	13	and	name	HN)	(resid	13	and	name	HA)	2.4	0.6	0.6
assign	(resid	13	and	name	HN)	(resid	13	and	name	HB*)	2.9	1.1	1.1
assign	(resid	13	and	name	HN)	(resid	13	and	name	HD*)	3.4	1.6	1.6
assign	(resid	13	and	name	HN)	(resid	13	and	name	HG*)	3.4	1.6	1.6
assign	(resid	13	and	name	HN)	(resid	13	and	name	HE*)	3.4	1.6	1.6

!LEU

assign	(resid	14	and	name	HB1)	(resid	14	and	name	HA)	2.9	1.1	1.1
assign	(resid	14	and	name	HB2)	(resid	14	and	name	HA)	2.4	0.6	0.6
assign	(resid	14	and	name	HD2*)	(resid	14	and	name	HA)	3.4	1.6	1.6
assign	(resid	14	and	name	HD2*)	(resid	14	and	name	HB1)	2.9	1.1	1.1
assign	(resid	14	and	name	HD2*)	(resid	14	and	name	HG)	2.4	0.6	0.6
assign	(resid	14	and	name	HD2*)	(resid	14	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	14	and	name	HD1*)	(resid	14	and	name	HB2)	2.9	1.1	1.1

assign	(resid	14	and	name	HD1*)	(resid	14	and	name	HG)	2.4	0.6	0.6
assign	(resid	14	and	name	HD1*)	(resid	14	and	name	HA)	2.9	1.1	1.1
assign	(resid	14	and	name	HG)	(resid	14	and	name	HA)	3.4	1.6	1.6
assign	(resid	14	and	name	HG)	(resid	14	and	name	HB1)	2.9	1.1	1.1
assign	(resid	14	and	name	HG)	(resid	14	and	name	HB2)	2.9	1.1	1.1
assign	(resid	14	and	name	HN)	(resid	14	and	name	HA)	2.9	1.1	1.1
assign	(resid	14	and	name	HN)	(resid	14	and	name	HB1)	2.4	0.6	0.6
assign	(resid	14	and	name	HN)	(resid	14	and	name	HB2)	2.4	0.6	0.6
assign	(resid	14	and	name	HN)	(resid	14	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	14	and	name	HN)	(resid	14	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	14	and	name	HN)	(resid	14	and	name	HG)	3.4	1.6	1.6

!ALA

assign	(resid	15	and	name	HB*)	(resid	15	and	name	HA)	2.4	0.6	0.6
assign	(resid	15	and	name	HN)	(resid	15	and	name	HA)	2.4	0.6	0.6
assign	(resid	15	and	name	HN)	(resid	15	and	name	HB*)	2.4	0.6	0.6

!LEU

assign	(resid	16	and	name	HB1)	(resid	16	and	name	HA)	2.9	1.1	1.1
assign	(resid	16	and	name	HB2)	(resid	16	and	name	HA)	2.4	0.6	0.6
assign	(resid	16	and	name	HG)	(resid	16	and	name	HA)	3.9	2.1	2.1
assign	(resid	16	and	name	HD1*)	(resid	16	and	name	HA)	3.4	1.6	1.6
assign	(resid	16	and	name	HD1*)	(resid	16	and	name	HB1)	2.9	1.1	1.1
assign	(resid	16	and	name	HD1*)	(resid	16	and	name	HG)	2.4	0.6	0.6
assign	(resid	16	and	name	HD2*)	(resid	16	and	name	HA)	2.9	1.1	1.1
assign	(resid	16	and	name	HD2*)	(resid	16	and	name	HB2)	2.9	1.1	1.1
assign	(resid	16	and	name	HD2*)	(resid	16	and	name	HG)	2.4	0.6	0.6
assign	(resid	16	and	name	HN)	(resid	16	and	name	HA)	2.4	0.6	0.6
assign	(resid	16	and	name	HN)	(resid	16	and	name	HB1)	2.4	0.6	0.6
assign	(resid	16	and	name	HN)	(resid	16	and	name	HB2)	2.4	0.6	0.6
assign	(resid	16	and	name	HN)	(resid	16	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	16	and	name	HN)	(resid	16	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	16	and	name	HN)	(resid	16	and	name	HG)	2.9	1.1	1.1

!VAL

assign	(resid	17	and	name	HA)	(resid	17	and	name	HB)	2.9	1.1	1.1
assign	(resid	17	and	name	HG2*)	(resid	17	and	name	HA)	2.4	0.6	0.6
assign	(resid	17	and	name	HG2*)	(resid	17	and	name	HB)	2.9	1.1	1.1
assign	(resid	17	and	name	HG2*)	(resid	17	and	name	HG1*)	2.4	0.6	0.6
assign	(resid	17	and	name	HG2*)	(resid	17	and	name	HN)	2.4	0.6	0.6
assign	(resid	17	and	name	HG1*)	(resid	17	and	name	HA)	2.4	0.6	0.6
assign	(resid	17	and	name	HG1*)	(resid	17	and	name	HB)	2.4	0.6	0.6
assign	(resid	17	and	name	HG1*)	(resid	17	and	name	HN)	3.4	1.6	1.6
assign	(resid	17	and	name	HN)	(resid	17	and	name	HA)	2.9	1.1	1.1
assign	(resid	17	and	name	HN)	(resid	17	and	name	HB)	2.4	0.6	0.6

!ILE

assign	(resid	18	and	name	HA)	(resid	18	and	name	HB)	2.9	1.1	1.1
assign	(resid	18	and	name	HA)	(resid	18	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	18	and	name	HA)	(resid	18	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	18	and	name	HD1*)	(resid	18	and	name	HA)	2.9	1.1	1.1
assign	(resid	18	and	name	HD1*)	(resid	18	and	name	HB)	3.4	1.6	1.6
assign	(resid	18	and	name	HG1*)	(resid	18	and	name	HB)	2.9	1.1	1.1
assign	(resid	18	and	name	HG1*)	(resid	18	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	18	and	name	HG2*)	(resid	18	and	name	HB)	2.4	0.6	0.6
assign	(resid	18	and	name	HG2*)	(resid	18	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	18	and	name	HG2*)	(resid	18	and	name	HG1*)	2.4	0.6	0.6
assign	(resid	18	and	name	HN)	(resid	18	and	name	HA)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	18	and	name	HB)	2.4	0.6	0.6
assign	(resid	18	and	name	HN)	(resid	18	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	18	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	18	and	name	HN)	(resid	18	and	name	HG2*)	3.4	1.6	1.6

!LYS

assign	(resid	19	and	name	HA)	(resid	19	and	name	HN)	2.4	0.6	0.6
assign	(resid	19	and	name	HB2)	(resid	19	and	name	HA)	2.4	0.6	0.6
assign	(resid	19	and	name	HB1)	(resid	19	and	name	HA)	2.4	0.6	0.6
assign	(resid	19	and	name	HB1)	(resid	19	and	name	HN)	2.4	0.6	0.6
assign	(resid	19	and	name	HG*)	(resid	19	and	name	HA)	2.9	1.1	1.1
assign	(resid	19	and	name	HG*)	(resid	19	and	name	HB*)	2.4	0.6	0.6
assign	(resid	19	and	name	HG*)	(resid	19	and	name	HN)	2.9	1.1	1.1
assign	(resid	19	and	name	HG*)	(resid	19	and	name	HE*)	2.4	0.6	0.6
assign	(resid	19	and	name	HG*)	(resid	19	and	name	HD*)	2.9	1.1	1.1

!SER

assign	(resid	20	and	name	HB1)	(resid	20	and	name	HA)	2.4	0.6	0.6
assign	(resid	20	and	name	HB2)	(resid	20	and	name	HA)	2.4	0.6	0.6
assign	(resid	20	and	name	HN)	(resid	20	and	name	HA)	2.9	1.1	1.1
assign	(resid	20	and	name	HN)	(resid	20	and	name	HB1)	2.9	1.1	1.1
assign	(resid	20	and	name	HN)	(resid	20	and	name	HB2)	3.4	1.6	1.6

!GLY

assign	(resid	21	and	name	HN)	(resid	21	and	name	HA*)	2.4	0.6	0.6

!LYS

assign	(resid	22	and	name	HA)	(resid	22	and	name	HE*)	3.9	2.1	2.1
assign	(resid	22	and	name	HB1)	(resid	22	and	name	HA)	2.4	0.6	0.6
assign	(resid	22	and	name	HB2)	(resid	22	and	name	HA)	2.9	1.1	1.1
assign	(resid	22	and	name	HB1)	(resid	22	and	name	HG*)	2.4	0.6	0.6
assign	(resid	22	and	name	HB2)	(resid	22	and	name	HG*)	2.9	1.1	1.1
assign	(resid	22	and	name	HE*)	(resid	22	and	name	HD*)	2.9	1.1	1.1
assign	(resid	22	and	name	HE*)	(resid	22	and	name	HN)	3.9	2.1	2.1
assign	(resid	22	and	name	HG*)	(resid	22	and	name	HA)	3.9	2.1	2.1
assign	(resid	22	and	name	HG*)	(resid	22	and	name	HD*)	2.4	0.6	0.6
assign	(resid	22	and	name	HG*)	(resid	22	and	name	HE*)	2.4	0.6	0.6
assign	(resid	22	and	name	HN)	(resid	22	and	name	HA)	3.4	1.6	1.6
assign	(resid	22	and	name	HN)	(resid	22	and	name	HB1)	3.4	1.6	1.6
assign	(resid	22	and	name	HN)	(resid	22	and	name	HB2)	2.9	1.1	1.1
assign	(resid	22	and	name	HN)	(resid	22	and	name	HG*)	3.9	2.1	2.1

!TYR

assign	(resid	23	and	name	HA)	(resid	23	and	name	HB1)	2.4	0.6	0.6
assign	(resid	23	and	name	HA)	(resid	23	and	name	HB2)	2.4	0.6	0.6
assign	(resid	23	and	name	HD1)	(resid	23	and	name	HA)	3.4	1.6	1.6
assign	(resid	23	and	name	HD1)	(resid	23	and	name	HB1)	2.4	0.6	0.6
assign	(resid	23	and	name	HD2)	(resid	23	and	name	HA)	3.4	1.6	1.6
assign	(resid	23	and	name	HD2)	(resid	23	and	name	HB2)	2.4	0.6	0.6
assign	(resid	23	and	name	HE1)	(resid	23	and	name	HB1)	3.4	1.6	1.6
assign	(resid	23	and	name	HE1)	(resid	23	and	name	HD1)	2.4	0.6	0.6
assign	(resid	23	and	name	HE2)	(resid	23	and	name	HB2)	3.4	1.6	1.6
assign	(resid	23	and	name	HE2)	(resid	23	and	name	HD2)	2.4	0.6	0.6
assign	(resid	23	and	name	HN)	(resid	23	and	name	HA)	3.4	1.6	1.6
assign	(resid	23	and	name	HN)	(resid	23	and	name	HB1)	3.4	1.6	1.6
assign	(resid	23	and	name	HN)	(resid	23	and	name	HD1)	2.9	1.1	1.1
assign	(resid	23	and	name	HN)	(resid	23	and	name	HE1)	3.9	2.1	2.1

!THR

assign	(resid	24	and	name	HB)	(resid	24	and	name	HA)	2.4	0.6	0.6
assign	(resid	24	and	name	HG2*)	(resid	24	and	name	HA)	2.9	1.1	1.1
assign	(resid	24	and	name	HG2*)	(resid	24	and	name	HB)	2.4	0.6	0.6
assign	(resid	24	and	name	HN)	(resid	24	and	name	HA)	2.9	1.1	1.1
assign	(resid	24	and	name	HN)	(resid	24	and	name	HB)	2.9	1.1	1.1
assign	(resid	24	and	name	HN)	(resid	24	and	name	HG2*)	2.4	0.6	0.6

!LEU

assign	(resid	25	and	name	HA)	(resid	25	and	name	HB1)	2.9	1.1	1.1
assign	(resid	25	and	name	HA)	(resid	25	and	name	HB2)	2.9	1.1	1.1
assign	(resid	25	and	name	HD1*)	(resid	25	and	name	HA)	3.9	2.1	2.1
assign	(resid	25	and	name	HD1*)	(resid	25	and	name	HB*)	3.4	1.6	1.6
assign	(resid	25	and	name	HD1*)	(resid	25	and	name	HG)	2.4	0.6	0.6
assign	(resid	25	and	name	HD1*)	(resid	25	and	name	HN)	3.4	1.6	1.6
assign	(resid	25	and	name	HD2*)	(resid	25	and	name	HA)	2.9	1.1	1.1
assign	(resid	25	and	name	HD2*)	(resid	25	and	name	HB*)	3.4	1.6	1.6
assign	(resid	25	and	name	HD2*)	(resid	25	and	name	HG)	2.4	0.6	0.6
assign	(resid	25	and	name	HD2*)	(resid	25	and	name	HN)	3.9	2.1	2.1
assign	(resid	25	and	name	HG)	(resid	25	and	name	HA)	3.4	1.6	1.6
assign	(resid	25	and	name	HN)	(resid	25	and	name	HA)	3.9	2.1	2.1
assign	(resid	25	and	name	HN)	(resid	25	and	name	HB1)	2.9	1.1	1.1
assign	(resid	25	and	name	HN)	(resid	25	and	name	HB2)	2.4	0.6	0.6
assign	(resid	25	and	name	HN)	(resid	25	and	name	HG*)	2.9	1.1	1.1

!GLY

assign	(resid	26	and	name	HN)	(resid	26	and	name	HA*)	2.9	1.1	1.1

!TYR

assign	(resid	27	and	name	HD1)	(resid	27	and	name	HB2)	2.9	1.1	1.1
assign	(resid	27	and	name	HD1)	(resid	27	and	name	HA)	2.9	1.1	1.1
assign	(resid	27	and	name	HD2)	(resid	27	and	name	HB1)	2.9	1.1	1.1
assign	(resid	27	and	name	HD2)	(resid	27	and	name	HA)	3.4	1.6	1.6
assign	(resid	27	and	name	HE2)	(resid	27	and	name	HB1)	3.9	2.1	2.1
assign	(resid	27	and	name	HE1)	(resid	27	and	name	HB2)	3.9	2.1	2.1
assign	(resid	27	and	name	HE1)	(resid	27	and	name	HA)	3.9	2.1	2.1
assign	(resid	27	and	name	HN)	(resid	27	and	name	HA)	2.9	1.1	1.1
assign	(resid	27	and	name	HN)	(resid	27	and	name	HB1)	3.9	2.1	2.1
assign	(resid	27	and	name	HN)	(resid	27	and	name	HB2)	2.9	1.1	1.1
assign	(resid	27	and	name	HN)	(resid	27	and	name	HD1)	3.9	2.1	2.1

!LYS

assign	(resid	28	and	name	HA)	(resid	28	and	name	HB*)	2.9	1.1	1.1
assign	(resid	28	and	name	HA)	(resid	28	and	name	HD*)	3.9	2.1	2.1
assign	(resid	28	and	name	HG*)	(resid	28	and	name	HA)	3.9	2.1	2.1
assign	(resid	28	and	name	HG*)	(resid	28	and	name	HD*)	3.9	2.1	2.1
assign	(resid	28	and	name	HN)	(resid	28	and	name	HA)	2.9	1.1	1.1
assign	(resid	28	and	name	HN)	(resid	28	and	name	HB*)	3.4	1.6	1.6
assign	(resid	28	and	name	HN)	(resid	28	and	name	HD*)	3.9	2.1	2.1
assign	(resid	28	and	name	HN)	(resid	28	and	name	HG*)	3.9	2.1	2.1

!SER

assign	(resid	29	and	name	HA)	(resid	29	and	name	HB*)	2.9	1.1	1.1

assign	(resid	29	and	name	HN	)	(resid	29	and	name	HA	)	2.4	0.6	0.6
assign	(resid	29	and	name	HN	)	(resid	29	and	name	HB*	)	2.9	1.1	1.1

!THR

assign	(resid	30	and	name	HA	)	(resid	30	and	name	HG2*	)	2.9	1.1	1.1
assign	(resid	30	and	name	HB	)	(resid	30	and	name	HA	)	2.4	0.6	0.6
assign	(resid	30	and	name	HB	)	(resid	30	and	name	HG2*	)	2.4	0.6	0.6
assign	(resid	30	and	name	HN	)	(resid	30	and	name	HA	)	3.4	1.6	1.6
assign	(resid	30	and	name	HN	)	(resid	30	and	name	HB	)	2.9	1.1	1.1
assign	(resid	30	and	name	HN	)	(resid	30	and	name	HG2*	)	2.4	0.6	0.6

!VAL

assign	(resid	31	and	name	HA	)	(resid	31	and	name	HB	)	3.4	1.6	1.6
assign	(resid	31	and	name	HG1*	)	(resid	31	and	name	HA	)	2.9	1.1	1.1
assign	(resid	31	and	name	HG1*	)	(resid	31	and	name	HB	)	2.4	0.6	0.6
assign	(resid	31	and	name	HG1*	)	(resid	31	and	name	HN	)	3.4	1.6	1.6
assign	(resid	31	and	name	HG1*	)	(resid	31	and	name	HG2*	)	2.4	0.6	0.6
assign	(resid	31	and	name	HG2*	)	(resid	31	and	name	HA	)	2.9	1.1	1.1
assign	(resid	31	and	name	HG2*	)	(resid	31	and	name	HB	)	2.4	0.6	0.6
assign	(resid	31	and	name	HG2*	)	(resid	31	and	name	HN	)	2.9	1.1	1.1
assign	(resid	31	and	name	HN	)	(resid	31	and	name	HA	)	3.4	1.6	1.6
assign	(resid	31	and	name	HN	)	(resid	31	and	name	HB	)	2.4	0.6	0.6

!LYS

assign	(resid	32	and	name	HA	)	(resid	32	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	32	and	name	HB*	)	(resid	32	and	name	HA	)	2.9	1.1	1.1
assign	(resid	32	and	name	HD*	)	(resid	32	and	name	HA	)	3.9	2.1	2.1
assign	(resid	32	and	name	HD*	)	(resid	32	and	name	HB*	)	3.4	1.6	1.6
assign	(resid	32	and	name	HD*	)	(resid	32	and	name	HG*	)	2.4	0.6	0.6
assign	(resid	32	and	name	HG*	)	(resid	32	and	name	HE*	)	3.9	2.1	2.1
assign	(resid	32	and	name	HN	)	(resid	32	and	name	HA	)	2.4	0.6	0.6
assign	(resid	32	and	name	HN	)	(resid	32	and	name	HB*	)	2.4	0.6	0.6
assign	(resid	32	and	name	HN	)	(resid	32	and	name	HE*	)	3.9	2.1	2.1
assign	(resid	32	and	name	HN	)	(resid	32	and	name	HG*	)	2.9	1.1	1.1

!SER

assign	(resid	33	and	name	HB1	)	(resid	33	and	name	HA	)	2.4	0.6	0.6
assign	(resid	33	and	name	HB2	)	(resid	33	and	name	HA	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	33	and	name	HA	)	2.4	0.6	0.6
assign	(resid	33	and	name	HN	)	(resid	33	and	name	HB1	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	33	and	name	HB2	)	2.4	0.6	0.6

!LEU

assign	(resid	34	and	name	HA	)	(resid	34	and	name	HB2	)	2.9	1.1	1.1
assign	(resid	34	and	name	HA	)	(resid	34	and	name	HB1	)	2.4	0.6	0.6
assign	(resid	34	and	name	HA	)	(resid	34	and	name	HG	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD1*	)	(resid	34	and	name	HA	)	3.4	1.6	1.6
assign	(resid	34	and	name	HD1*	)	(resid	34	and	name	HB*	)	2.4	0.6	0.6
assign	(resid	34	and	name	HD1*	)	(resid	34	and	name	HG	)	2.4	0.6	0.6
assign	(resid	34	and	name	HD1*	)	(resid	34	and	name	HN	)	3.4	1.6	1.6
assign	(resid	34	and	name	HD2*	)	(resid	34	and	name	HA	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD2*	)	(resid	34	and	name	HB*	)	2.4	0.6	0.6
assign	(resid	34	and	name	HD2*	)	(resid	34	and	name	HG	)	2.4	0.6	0.6
assign	(resid	34	and	name	HD2*	)	(resid	34	and	name	HN	)	3.9	2.1	2.1
assign	(resid	34	and	name	HN	)	(resid	34	and	name	HA	)	2.9	1.1	1.1
assign	(resid	34	and	name	HN	)	(resid	34	and	name	HB2	)	2.4	0.6	0.6
assign	(resid	34	and	name	HN	)	(resid	34	and	name	HB1	)	2.9	1.1	1.1
assign	(resid	34	and	name	HN	)	(resid	34	and	name	HG	)	2.9	1.1	1.1

!ARG

assign	(resid	35	and	name	HA	)	(resid	35	and	name	HB1	)	2.4	0.6	0.6
assign	(resid	35	and	name	HA	)	(resid	35	and	name	HB2	)	2.9	1.1	1.1
assign	(resid	35	and	name	HA	)	(resid	35	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	35	and	name	HD*	)	(resid	35	and	name	HB*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HD*	)	(resid	35	and	name	HH*	)	3.9	2.1	2.1
assign	(resid	35	and	name	HE	)	(resid	35	and	name	HB*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HE	)	(resid	35	and	name	HD*	)	2.4	0.6	0.6
assign	(resid	35	and	name	HE	)	(resid	35	and	name	HG*	)	2.9	1.1	1.1
assign	(resid	35	and	name	HE	)	(resid	35	and	name	HH*	)	2.4	0.6	0.6
assign	(resid	35	and	name	HG*	)	(resid	35	and	name	HD*	)	2.4	0.6	0.6
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HA	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HB2	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HB1	)	3.4	1.6	1.6
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HD*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HE	)	3.9	2.1	2.1
assign	(resid	35	and	name	HN	)	(resid	35	and	name	HG*	)	3.4	1.6	1.6

!GLN

assign	(resid	36	and	name	HA	)	(resid	36	and	name	HB1	)	2.9	1.1	1.1
assign	(resid	36	and	name	HA	)	(resid	36	and	name	HB2	)	3.4	1.6	1.6
assign	(resid	36	and	name	HE2*	)	(resid	36	and	name	HA	)	3.4	1.6	1.6
assign	(resid	36	and	name	HE2*	)	(resid	36	and	name	HB2	)	3.4	1.6	1.6
assign	(resid	36	and	name	HE2*	)	(resid	36	and	name	HB1	)	3.4	1.6	1.6
assign	(resid	36	and	name	HG*	)	(resid	36	and	name	HA	)	3.9	2.1	2.1

assign	(resid	36	and	name	HG*)	(resid	36	and	name	HB1)	2.9	1.1	1.1
assign	(resid	36	and	name	HG*)	(resid	36	and	name	HB2)	2.9	1.1	1.1
assign	(resid	36	and	name	HG*)	(resid	36	and	name	HE2*)	2.9	1.1	1.1
assign	(resid	36	and	name	HN)	(resid	36	and	name	HA)	2.4	0.6	0.6
assign	(resid	36	and	name	HN)	(resid	36	and	name	HB2)	2.4	0.6	0.6
assign	(resid	36	and	name	HN)	(resid	36	and	name	HB1)	2.9	1.1	1.1
assign	(resid	36	and	name	HN)	(resid	36	and	name	HE2*)	3.9	2.1	2.1
assign	(resid	36	and	name	HN)	(resid	36	and	name	HG*)	2.9	1.1	1.1

!GLY

assign	(resid	37	and	name	HN)	(resid	37	and	name	HA*)	2.4	0.6	0.6

!LYS

assign	(resid	38	and	name	HA)	(resid	38	and	name	HB*)	2.9	1.1	1.1
assign	(resid	38	and	name	HA)	(resid	38	and	name	HG*)	2.9	1.1	1.1
assign	(resid	38	and	name	HB*)	(resid	38	and	name	HG*)	3.4	1.6	1.6
assign	(resid	38	and	name	HN)	(resid	38	and	name	HA)	2.4	0.6	0.6
assign	(resid	38	and	name	HN)	(resid	38	and	name	HB*)	2.9	1.1	1.1
assign	(resid	38	and	name	HN)	(resid	38	and	name	HE*)	3.9	2.1	2.1
assign	(resid	38	and	name	HN)	(resid	38	and	name	HG*)	3.4	1.6	1.6

!SER

assign	(resid	39	and	name	HB2)	(resid	39	and	name	HA)	2.4	0.6	0.6
assign	(resid	39	and	name	HB1)	(resid	39	and	name	HA)	2.4	0.6	0.6
assign	(resid	39	and	name	HN)	(resid	39	and	name	HA)	2.4	0.6	0.6
assign	(resid	39	and	name	HN)	(resid	39	and	name	HB1)	2.4	0.6	0.6
assign	(resid	39	and	name	HN)	(resid	39	and	name	HB2)	2.9	1.1	1.1

!LYS

assign	(resid	40	and	name	HA)	(resid	40	and	name	HB*)	2.4	0.6	0.6
assign	(resid	40	and	name	HB*)	(resid	40	and	name	HE*)	2.4	0.6	0.6
assign	(resid	40	and	name	HB*)	(resid	40	and	name	HG*)	3.9	2.1	2.1
assign	(resid	40	and	name	HE*)	(resid	40	and	name	HG*)	2.4	0.6	0.6
assign	(resid	40	and	name	HG*)	(resid	40	and	name	HA)	3.9	2.1	2.1
assign	(resid	40	and	name	HN)	(resid	40	and	name	HA)	2.4	0.6	0.6
assign	(resid	40	and	name	HN)	(resid	40	and	name	HB*)	2.4	0.6	0.6
assign	(resid	40	and	name	HN)	(resid	40	and	name	HG*)	3.4	1.6	1.6

!LEU

assign	(resid	41	and	name	HB*)	(resid	41	and	name	HG)	2.4	0.6	0.6
assign	(resid	41	and	name	HD1*)	(resid	41	and	name	HA)	3.4	1.6	1.6
assign	(resid	41	and	name	HD1*)	(resid	41	and	name	HB*)	3.4	1.6	1.6
assign	(resid	41	and	name	HD1*)	(resid	41	and	name	HG)	2.4	0.6	0.6
assign	(resid	41	and	name	HD1*)	(resid	41	and	name	HD2*)	2.4	0.6	0.6
assign	(resid	41	and	name	HD2*)	(resid	41	and	name	HA)	2.9	1.1	1.1
assign	(resid	41	and	name	HD2*)	(resid	41	and	name	HB*)	3.4	1.6	1.6
assign	(resid	41	and	name	HD2*)	(resid	41	and	name	HG)	2.4	0.6	0.6
assign	(resid	41	and	name	HD2*)	(resid	41	and	name	HN)	3.9	2.1	2.1
assign	(resid	41	and	name	HN)	(resid	41	and	name	HA)	2.9	1.1	1.1
assign	(resid	41	and	name	HN)	(resid	41	and	name	HB*)	2.9	1.1	1.1
assign	(resid	41	and	name	HN)	(resid	41	and	name	HG)	3.4	1.6	1.6

!ILE

assign	(resid	42	and	name	HA)	(resid	42	and	name	HB)	2.4	0.6	0.6
assign	(resid	42	and	name	HA)	(resid	42	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	42	and	name	HA)	(resid	42	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	42	and	name	HA)	(resid	42	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	42	and	name	HG1*)	(resid	42	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	42	and	name	HG2*)	(resid	42	and	name	HB)	2.4	0.6	0.6
assign	(resid	42	and	name	HG2*)	(resid	42	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	42	and	name	HN)	(resid	42	and	name	HA)	3.9	2.1	2.1
assign	(resid	42	and	name	HN)	(resid	42	and	name	HB)	3.9	2.1	2.1
assign	(resid	42	and	name	HN)	(resid	42	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	42	and	name	HN)	(resid	42	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	42	and	name	HN)	(resid	42	and	name	HG2*)	2.9	1.1	1.1

!ILE

assign	(resid	43	and	name	HA)	(resid	43	and	name	HB)	3.4	1.6	1.6
assign	(resid	43	and	name	HA)	(resid	43	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	43	and	name	HA)	(resid	43	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	43	and	name	HD1*)	(resid	43	and	name	HB)	2.9	1.1	1.1
assign	(resid	43	and	name	HG1*)	(resid	43	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	43	and	name	HA)	2.4	0.6	0.6
assign	(resid	43	and	name	HG2*)	(resid	43	and	name	HB)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	43	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	43	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	43	and	name	HN)	3.9	2.1	2.1
assign	(resid	43	and	name	HN)	(resid	43	and	name	HA)	3.4	1.6	1.6
assign	(resid	43	and	name	HN)	(resid	43	and	name	HB)	2.4	0.6	0.6
assign	(resid	43	and	name	HN)	(resid	43	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	43	and	name	HN)	(resid	43	and	name	HG1*)	2.4	0.6	0.6
assign	(resid	43	and	name	HN)	(resid	43	and	name	HG2*)	3.4	1.6	1.6

!ILE

assign	(resid	44	and	name	HA)	(resid	44	and	name	HG2*)	2.9	1.1	1.1

assign	(resid	44	and	name	HA)	(resid	44	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	44	and	name	HA)	(resid	44	and	name	HB)	2.9	1.1	1.1
assign	(resid	44	and	name	HB)	(resid	44	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	44	and	name	HD1*)	(resid	44	and	name	HA)	3.9	2.1	2.1
assign	(resid	44	and	name	HD1*)	(resid	44	and	name	HG1*)	2.4	0.6	0.6
assign	(resid	44	and	name	HD1*)	(resid	44	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	44	and	name	HA)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	44	and	name	HB)	2.4	0.6	0.6
assign	(resid	44	and	name	HG2*)	(resid	44	and	name	HG1*)	2.4	0.6	0.6
assign	(resid	44	and	name	HN)	(resid	44	and	name	HA)	3.4	1.6	1.6
assign	(resid	44	and	name	HN)	(resid	44	and	name	HB)	2.9	1.1	1.1
assign	(resid	44	and	name	HN)	(resid	44	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	44	and	name	HN)	(resid	44	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	44	and	name	HN)	(resid	44	and	name	HG2*)	3.4	1.6	1.6

!ALA

assign	(resid	45	and	name	HB*)	(resid	45	and	name	HA)	2.4	0.6	0.6
assign	(resid	45	and	name	HN)	(resid	45	and	name	HA)	2.9	1.1	1.1
assign	(resid	45	and	name	HN)	(resid	45	and	name	HB*)	2.4	0.6	0.6

!ALA

assign	(resid	46	and	name	HA)	(resid	46	and	name	HB*)	2.4	0.6	0.6
assign	(resid	46	and	name	HN)	(resid	46	and	name	HA)	2.4	0.6	0.6
assign	(resid	46	and	name	HN)	(resid	46	and	name	HB*)	2.4	0.6	0.6

!ASN

assign	(resid	47	and	name	HB1)	(resid	47	and	name	HA)	2.9	1.1	1.1
assign	(resid	47	and	name	HB2)	(resid	47	and	name	HA)	2.9	1.1	1.1
assign	(resid	47	and	name	HD2*)	(resid	47	and	name	HA)	3.9	2.1	2.1
assign	(resid	47	and	name	HD2*)	(resid	47	and	name	HB1)	2.9	1.1	1.1
assign	(resid	47	and	name	HD2*)	(resid	47	and	name	HB2)	2.9	1.1	1.1
assign	(resid	47	and	name	HD2*)	(resid	47	and	name	HN)	3.9	2.1	2.1
assign	(resid	47	and	name	HN)	(resid	47	and	name	HA)	2.9	1.1	1.1
assign	(resid	47	and	name	HN)	(resid	47	and	name	HB1)	2.9	1.1	1.1
assign	(resid	47	and	name	HN)	(resid	47	and	name	HB2)	3.4	1.6	1.6

!THR

assign	(resid	48	and	name	HB)	(resid	48	and	name	HA)	2.9	1.1	1.1
assign	(resid	48	and	name	HG2*)	(resid	48	and	name	HA)	3.4	1.6	1.6
assign	(resid	48	and	name	HG2*)	(resid	48	and	name	HB)	2.9	1.1	1.1
assign	(resid	48	and	name	HN)	(resid	48	and	name	HA)	2.9	1.1	1.1
assign	(resid	48	and	name	HN)	(resid	48	and	name	HB)	2.4	0.6	0.6
assign	(resid	48	and	name	HN)	(resid	48	and	name	HG2*)	2.9	1.1	1.1

!PRO

assign	(resid	49	and	name	HA)	(resid	49	and	name	HB*)	2.9	1.1	1.1
assign	(resid	49	and	name	HA)	(resid	49	and	name	HD*)	3.9	2.1	2.1
assign	(resid	49	and	name	HA)	(resid	49	and	name	HG*)	2.9	1.1	1.1
assign	(resid	49	and	name	HD*)	(resid	49	and	name	HB*)	3.9	2.1	2.1
assign	(resid	49	and	name	HD*)	(resid	49	and	name	HG*)	3.4	1.6	1.6

!VAL

assign	(resid	50	and	name	HB)	(resid	50	and	name	HA)	2.9	1.1	1.1
assign	(resid	50	and	name	HG2*)	(resid	50	and	name	HA)	2.9	1.1	1.1
assign	(resid	50	and	name	HG1*)	(resid	50	and	name	HA)	2.9	1.1	1.1
assign	(resid	50	and	name	HG2*)	(resid	50	and	name	HB)	2.4	0.6	0.6
assign	(resid	50	and	name	HG1*)	(resid	50	and	name	HB)	2.4	0.6	0.6
assign	(resid	50	and	name	HN)	(resid	50	and	name	HA)	2.4	0.6	0.6
assign	(resid	50	and	name	HN)	(resid	50	and	name	HB)	2.9	1.1	1.1
assign	(resid	50	and	name	HN)	(resid	50	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	50	and	name	HN)	(resid	50	and	name	HG1*)	3.4	1.6	1.6

!LEU

assign	(resid	51	and	name	HB2)	(resid	51	and	name	HA)	2.9	1.1	1.1
assign	(resid	51	and	name	HB1)	(resid	51	and	name	HA)	2.4	0.6	0.6
assign	(resid	51	and	name	HD1*)	(resid	51	and	name	HA)	2.9	1.1	1.1
assign	(resid	51	and	name	HD1*)	(resid	51	and	name	HB*)	2.9	1.1	1.1
assign	(resid	51	and	name	HD1*)	(resid	51	and	name	HG)	2.4	0.6	0.6
assign	(resid	51	and	name	HD1*)	(resid	51	and	name	HN)	2.9	1.1	1.1
assign	(resid	51	and	name	HD2*)	(resid	51	and	name	HA)	3.4	1.6	1.6
assign	(resid	51	and	name	HD2*)	(resid	51	and	name	HB*)	2.9	1.1	1.1
assign	(resid	51	and	name	HD2*)	(resid	51	and	name	HG)	2.4	0.6	0.6
assign	(resid	51	and	name	HD2*)	(resid	51	and	name	HN)	2.9	1.1	1.1
assign	(resid	51	and	name	HG)	(resid	51	and	name	HA)	3.9	2.1	2.1
assign	(resid	51	and	name	HG)	(resid	51	and	name	HB*)	2.9	1.1	1.1
assign	(resid	51	and	name	HN)	(resid	51	and	name	HA)	2.4	0.6	0.6
assign	(resid	51	and	name	HN)	(resid	51	and	name	HB1)	2.9	1.1	1.1
assign	(resid	51	and	name	HN)	(resid	51	and	name	HB2)	2.4	0.6	0.6
assign	(resid	51	and	name	HN)	(resid	51	and	name	HG)	3.4	1.6	1.6

!ARG

assign	(resid	52	and	name	HA)	(resid	52	and	name	HB2)	2.4	0.6	0.6
assign	(resid	52	and	name	HA)	(resid	52	and	name	HB1)	2.9	1.1	1.1
assign	(resid	52	and	name	HD*)	(resid	52	and	name	HB*)	3.9	2.1	2.1
assign	(resid	52	and	name	HE)	(resid	52	and	name	HA)	3.4	1.6	1.6

assign	(resid	52	and	name	HE)	(resid	52	and	name	HD*)	2.4	0.6	0.6
assign	(resid	52	and	name	HE)	(resid	52	and	name	HG*)	2.9	1.1	1.1
assign	(resid	52	and	name	HG*)	(resid	52	and	name	HB*)	2.9	1.1	1.1
assign	(resid	52	and	name	HG*)	(resid	52	and	name	HD*)	2.9	1.1	1.1
assign	(resid	52	and	name	HN)	(resid	52	and	name	HA)	2.4	0.6	0.6
assign	(resid	52	and	name	HN)	(resid	52	and	name	HB1)	2.9	1.1	1.1
assign	(resid	52	and	name	HN)	(resid	52	and	name	HB2)	2.4	0.6	0.6
assign	(resid	52	and	name	HN)	(resid	52	and	name	HD*)	3.9	2.1	2.1
assign	(resid	52	and	name	HN)	(resid	52	and	name	HE)	3.9	2.1	2.1
assign	(resid	52	and	name	HN)	(resid	52	and	name	HG*)	3.4	1.6	1.6

!LYS

assign	(resid	53	and	name	HA)	(resid	53	and	name	HD*)	3.9	2.1	2.1
assign	(resid	53	and	name	HA)	(resid	53	and	name	HG*)	3.9	2.1	2.1
assign	(resid	53	and	name	HB1)	(resid	53	and	name	HA)	2.4	0.6	0.6
assign	(resid	53	and	name	HB2)	(resid	53	and	name	HA)	2.4	0.6	0.6
assign	(resid	53	and	name	HD*)	(resid	53	and	name	HB*)	2.4	0.6	0.6
assign	(resid	53	and	name	HE*)	(resid	53	and	name	HB*)	3.9	2.1	2.1
assign	(resid	53	and	name	HE*)	(resid	53	and	name	HD*)	2.4	0.6	0.6
assign	(resid	53	and	name	HG*)	(resid	53	and	name	HB*)	2.9	1.1	1.1
assign	(resid	53	and	name	HG*)	(resid	53	and	name	HD*)	2.9	1.1	1.1
assign	(resid	53	and	name	HG*)	(resid	53	and	name	HE*)	3.4	1.6	1.6
assign	(resid	53	and	name	HN)	(resid	53	and	name	HA)	2.9	1.1	1.1
assign	(resid	53	and	name	HN)	(resid	53	and	name	HB1)	2.4	0.6	0.6
assign	(resid	53	and	name	HN)	(resid	53	and	name	HB2)	2.9	1.1	1.1
assign	(resid	53	and	name	HN)	(resid	53	and	name	HD*)	3.4	1.6	1.6
assign	(resid	53	and	name	HN)	(resid	53	and	name	HE*)	3.9	2.1	2.1
assign	(resid	53	and	name	HN)	(resid	53	and	name	HG*)	3.4	1.6	1.6

!SER

assign	(resid	54	and	name	HB*)	(resid	54	and	name	HA)	2.4	0.6	0.6
assign	(resid	54	and	name	HN)	(resid	54	and	name	HA)	2.9	1.1	1.1
assign	(resid	54	and	name	HN)	(resid	54	and	name	HB*)	2.4	0.6	0.6

!GLU

assign	(resid	55	and	name	HA)	(resid	55	and	name	HB*)	2.4	0.6	0.6
assign	(resid	55	and	name	HB*)	(resid	55	and	name	HG*)	2.4	0.6	0.6
assign	(resid	55	and	name	HG*)	(resid	55	and	name	HA)	2.9	1.1	1.1
assign	(resid	55	and	name	HN)	(resid	55	and	name	HA)	2.4	0.6	0.6
assign	(resid	55	and	name	HN)	(resid	55	and	name	HB*)	2.9	1.1	1.1
assign	(resid	55	and	name	HN)	(resid	55	and	name	HG*)	3.4	1.6	1.6

!LEU

assign	(resid	56	and	name	HA)	(resid	56	and	name	HB*)	2.9	1.1	1.1
assign	(resid	56	and	name	HD1*)	(resid	56	and	name	HA)	3.9	2.1	2.1
assign	(resid	56	and	name	HD1*)	(resid	56	and	name	HN)	3.4	1.6	1.6
assign	(resid	56	and	name	HD1*)	(resid	56	and	name	HB*)	3.4	1.6	1.6
assign	(resid	56	and	name	HD1*)	(resid	56	and	name	HG)	2.4	0.6	0.6
assign	(resid	56	and	name	HD1*)	(resid	56	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	56	and	name	HD2*)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	56	and	name	HD2*)	(resid	56	and	name	HN)	2.9	1.1	1.1
assign	(resid	56	and	name	HD2*)	(resid	56	and	name	HB*)	3.4	1.6	1.6
assign	(resid	56	and	name	HD2*)	(resid	56	and	name	HG)	2.4	0.6	0.6
assign	(resid	56	and	name	HG)	(resid	56	and	name	HA)	3.4	1.6	1.6
assign	(resid	56	and	name	HG)	(resid	56	and	name	HB*)	3.9	2.1	2.1
assign	(resid	56	and	name	HN)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	56	and	name	HN)	(resid	56	and	name	HB*)	3.4	1.6	1.6
assign	(resid	56	and	name	HN)	(resid	56	and	name	HG)	3.4	1.6	1.6

!GLU

assign	(resid	57	and	name	HA)	(resid	57	and	name	HG*)	3.9	2.1	2.1
assign	(resid	57	and	name	HB1)	(resid	57	and	name	HA)	2.9	1.1	1.1
assign	(resid	57	and	name	HB2)	(resid	57	and	name	HA)	2.4	0.6	0.6
assign	(resid	57	and	name	HB1)	(resid	57	and	name	HG*)	2.4	0.6	0.6
assign	(resid	57	and	name	HB2)	(resid	57	and	name	HG*)	2.4	0.6	0.6
assign	(resid	57	and	name	HN)	(resid	57	and	name	HA)	2.4	0.6	0.6
assign	(resid	57	and	name	HN)	(resid	57	and	name	HB1)	2.4	0.6	0.6
assign	(resid	57	and	name	HN)	(resid	57	and	name	HB2)	2.4	0.6	0.6
assign	(resid	57	and	name	HN)	(resid	57	and	name	HG*)	2.9	1.1	1.1

!TYR

assign	(resid	58	and	name	HA)	(resid	58	and	name	HB1)	2.9	1.1	1.1
assign	(resid	58	and	name	HA)	(resid	58	and	name	HB2)	2.4	0.6	0.6
assign	(resid	58	and	name	HA)	(resid	58	and	name	HD1)	2.9	1.1	1.1
assign	(resid	58	and	name	HB2)	(resid	58	and	name	HD1)	2.9	1.1	1.1
assign	(resid	58	and	name	HB2)	(resid	58	and	name	HE1)	3.4	1.6	1.6
assign	(resid	58	and	name	HB1)	(resid	58	and	name	HD2)	2.9	1.1	1.1
assign	(resid	58	and	name	HB1)	(resid	58	and	name	HE2)	3.4	1.6	1.6
assign	(resid	58	and	name	HN)	(resid	58	and	name	HA)	2.9	1.1	1.1
assign	(resid	58	and	name	HN)	(resid	58	and	name	HB1)	2.4	0.6	0.6
assign	(resid	58	and	name	HN)	(resid	58	and	name	HB2)	2.4	0.6	0.6
assign	(resid	58	and	name	HN)	(resid	58	and	name	HD1)	3.4	1.6	1.6

!TYR

assign	(resid	59	and	name	HA)	(resid	59	and	name	HB1)	2.4	0.6	0.6
assign	(resid	59	and	name	HA)	(resid	59	and	name	HB2)	2.9	1.1	1.1
assign	(resid	59	and	name	HA)	(resid	59	and	name	HD2)	2.9	1.1	1.1
assign	(resid	59	and	name	HA)	(resid	59	and	name	HE2)	3.9	2.1	2.1
assign	(resid	59	and	name	HB1)	(resid	59	and	name	HD1)	3.9	2.1	2.1
assign	(resid	59	and	name	HB2)	(resid	59	and	name	HD1)	3.9	2.1	2.1
assign	(resid	59	and	name	HB1)	(resid	59	and	name	HE1)	3.9	2.1	2.1
assign	(resid	59	and	name	HB2)	(resid	59	and	name	HE1)	3.9	2.1	2.1
assign	(resid	59	and	name	HN)	(resid	59	and	name	HA)	2.4	0.6	0.6
assign	(resid	59	and	name	HN)	(resid	59	and	name	HB1)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	59	and	name	HB2)	2.4	0.6	0.6
assign	(resid	59	and	name	HN)	(resid	59	and	name	HD1)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	59	and	name	HE1)	3.4	1.6	1.6

!ALA

assign	(resid	60	and	name	HB*)	(resid	60	and	name	HA)	2.4	0.6	0.6
assign	(resid	60	and	name	HN)	(resid	60	and	name	HA)	2.4	0.6	0.6
assign	(resid	60	and	name	HN)	(resid	60	and	name	HB*)	2.4	0.6	0.6

!MET

assign	(resid	61	and	name	HB1)	(resid	61	and	name	HA)	2.9	1.1	1.1
assign	(resid	61	and	name	HB2)	(resid	61	and	name	HA)	2.4	0.6	0.6
assign	(resid	61	and	name	HE*)	(resid	61	and	name	HA)	3.9	2.1	2.1
assign	(resid	61	and	name	HE*)	(resid	61	and	name	HB1)	3.9	2.1	2.1
assign	(resid	61	and	name	HE*)	(resid	61	and	name	HB2)	3.9	2.1	2.1
assign	(resid	61	and	name	HG*)	(resid	61	and	name	HA)	2.9	1.1	1.1
assign	(resid	61	and	name	HG*)	(resid	61	and	name	HB1)	2.9	1.1	1.1
assign	(resid	61	and	name	HG*)	(resid	61	and	name	HB2)	2.9	1.1	1.1
assign	(resid	61	and	name	HG*)	(resid	61	and	name	HE*)	2.9	1.1	1.1
assign	(resid	61	and	name	HN)	(resid	61	and	name	HA)	2.4	0.6	0.6
assign	(resid	61	and	name	HN)	(resid	61	and	name	HB1)	2.4	0.6	0.6
assign	(resid	61	and	name	HN)	(resid	61	and	name	HB2)	2.4	0.6	0.6
assign	(resid	61	and	name	HN)	(resid	61	and	name	HE*)	3.4	1.6	1.6
assign	(resid	61	and	name	HN)	(resid	61	and	name	HG*)	3.4	1.6	1.6

!LEU

assign	(resid	62	and	name	HB1)	(resid	62	and	name	HA)	2.4	0.6	0.6
assign	(resid	62	and	name	HB2)	(resid	62	and	name	HA)	2.9	1.1	1.1
assign	(resid	62	and	name	HD1*)	(resid	62	and	name	HA)	3.4	1.6	1.6
assign	(resid	62	and	name	HD1*)	(resid	62	and	name	HB*)	2.9	1.1	1.1
assign	(resid	62	and	name	HD1*)	(resid	62	and	name	HG)	2.4	0.6	0.6
assign	(resid	62	and	name	HD1*)	(resid	62	and	name	HN)	3.4	1.6	1.6
assign	(resid	62	and	name	HD1*)	(resid	62	and	name	HD2*)	2.4	0.6	0.6
assign	(resid	62	and	name	HD2*)	(resid	62	and	name	HA)	2.4	0.6	0.6
assign	(resid	62	and	name	HD2*)	(resid	62	and	name	HB*)	2.4	0.6	0.6
assign	(resid	62	and	name	HD2*)	(resid	62	and	name	HG)	2.4	0.6	0.6
assign	(resid	62	and	name	HD2*)	(resid	62	and	name	HN)	3.4	1.6	1.6
assign	(resid	62	and	name	HG)	(resid	62	and	name	HA)	3.4	1.6	1.6
assign	(resid	62	and	name	HG)	(resid	62	and	name	HB*)	2.9	1.1	1.1
assign	(resid	62	and	name	HG)	(resid	62	and	name	HN)	2.4	0.6	0.6
assign	(resid	62	and	name	HN)	(resid	62	and	name	HA)	2.4	0.6	0.6
assign	(resid	62	and	name	HN)	(resid	62	and	name	HB2)	2.9	1.1	1.1
assign	(resid	62	and	name	HN)	(resid	62	and	name	HB1)	2.9	1.1	1.1

!SER

assign	(resid	63	and	name	HA)	(resid	63	and	name	HB*)	2.4	0.6	0.6
assign	(resid	63	and	name	HN)	(resid	63	and	name	HA)	2.4	0.6	0.6
assign	(resid	63	and	name	HN)	(resid	63	and	name	HB*)	2.4	0.6	0.6

!LYS

assign	(resid	64	and	name	HA)	(resid	64	and	name	HB*)	2.4	0.6	0.6
assign	(resid	64	and	name	HA)	(resid	64	and	name	HG*)	2.9	1.1	1.1
assign	(resid	64	and	name	HN)	(resid	64	and	name	HA)	2.4	0.6	0.6
assign	(resid	64	and	name	HN)	(resid	64	and	name	HB*)	2.9	1.1	1.1
assign	(resid	64	and	name	HN)	(resid	64	and	name	HG*)	2.9	1.1	1.1

!THR

assign	(resid	65	and	name	HB)	(resid	65	and	name	HA)	2.4	0.6	0.6
assign	(resid	65	and	name	HG2*)	(resid	65	and	name	HA)	2.9	1.1	1.1
assign	(resid	65	and	name	HG2*)	(resid	65	and	name	HB)	3.4	1.6	1.6
assign	(resid	65	and	name	HN)	(resid	65	and	name	HA)	2.4	0.6	0.6
assign	(resid	65	and	name	HN)	(resid	65	and	name	HB)	2.4	0.6	0.6
assign	(resid	65	and	name	HN)	(resid	65	and	name	HG2*)	3.4	1.6	1.6

!LYS

assign	(resid	66	and	name	HA)	(resid	66	and	name	HB*)	2.4	0.6	0.6
assign	(resid	66	and	name	HA)	(resid	66	and	name	HE*)	3.9	2.1	2.1
assign	(resid	66	and	name	HG*)	(resid	66	and	name	HA)	3.4	1.6	1.6
assign	(resid	66	and	name	HN)	(resid	66	and	name	HA)	2.9	1.1	1.1
assign	(resid	66	and	name	HN)	(resid	66	and	name	HB*)	2.9	1.1	1.1
assign	(resid	66	and	name	HN)	(resid	66	and	name	HE*)	3.9	2.1	2.1
assign	(resid	66	and	name	HN)	(resid	66	and	name	HG*)	2.9	1.1	1.1

!VAL

assign	(resid	67	and	name	HA)	(resid	67	and	name	HB)	3.4	1.6	1.6

assign	(resid	67	and	name	HG1*)	(resid	67	and	name	HA)	2.9	1.1	1.1
assign	(resid	67	and	name	HG1*)	(resid	67	and	name	HB)	2.4	0.6	0.6
assign	(resid	67	and	name	HG1*)	(resid	67	and	name	HN)	3.4	1.6	1.6
assign	(resid	67	and	name	HG1*)	(resid	67	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	67	and	name	HG2*)	(resid	67	and	name	HA)	2.9	1.1	1.1
assign	(resid	67	and	name	HG2*)	(resid	67	and	name	HB)	2.4	0.6	0.6
assign	(resid	67	and	name	HG2*)	(resid	67	and	name	HN)	2.4	0.6	0.6
assign	(resid	67	and	name	HN)	(resid	67	and	name	HA)	3.4	1.6	1.6
assign	(resid	67	and	name	HN)	(resid	67	and	name	HB)	2.9	1.1	1.1

!TYR

assign	(resid	68	and	name	HA)	(resid	68	and	name	HB1)	2.4	0.6	0.6
assign	(resid	68	and	name	HA)	(resid	68	and	name	HB2)	2.9	1.1	1.1
assign	(resid	68	and	name	HB1)	(resid	68	and	name	HD2)	2.9	1.1	1.1
assign	(resid	68	and	name	HB1)	(resid	68	and	name	HE2)	3.9	2.1	2.1
assign	(resid	68	and	name	HB2)	(resid	68	and	name	HD1)	2.9	1.1	1.1
assign	(resid	68	and	name	HB2)	(resid	68	and	name	HE1)	3.9	2.1	2.1
assign	(resid	68	and	name	HD1)	(resid	68	and	name	HA)	2.9	1.1	1.1
assign	(resid	68	and	name	HD2)	(resid	68	and	name	HA)	3.4	1.6	1.6
assign	(resid	68	and	name	HE1)	(resid	68	and	name	HA)	3.9	2.1	2.1
assign	(resid	68	and	name	HN)	(resid	68	and	name	HA)	3.4	1.6	1.6
assign	(resid	68	and	name	HN)	(resid	68	and	name	HB1)	2.9	1.1	1.1
assign	(resid	68	and	name	HN)	(resid	68	and	name	HB2)	2.9	1.1	1.1
assign	(resid	68	and	name	HN)	(resid	68	and	name	HD2)	3.4	1.6	1.6

!TYR

assign	(resid	69	and	name	HA)	(resid	69	and	name	HB1)	2.9	1.1	1.1
assign	(resid	69	and	name	HA)	(resid	69	and	name	HB2)	3.4	1.6	1.6
assign	(resid	69	and	name	HB1)	(resid	69	and	name	HE2)	3.4	1.6	1.6
assign	(resid	69	and	name	HB1)	(resid	69	and	name	HD2)	2.4	0.6	0.6
assign	(resid	69	and	name	HB2)	(resid	69	and	name	HD1)	2.4	0.6	0.6
assign	(resid	69	and	name	HE2)	(resid	69	and	name	HA)	3.9	2.1	2.1
assign	(resid	69	and	name	HD2)	(resid	69	and	name	HA)	2.9	1.1	1.1
assign	(resid	69	and	name	HN)	(resid	69	and	name	HA)	2.9	1.1	1.1
assign	(resid	69	and	name	HN)	(resid	69	and	name	HB1)	2.9	1.1	1.1
assign	(resid	69	and	name	HN)	(resid	69	and	name	HB2)	2.9	1.1	1.1
assign	(resid	69	and	name	HN)	(resid	69	and	name	HD1)	3.4	1.6	1.6

!PHE

assign	(resid	70	and	name	HA)	(resid	70	and	name	HB2)	2.4	0.6	0.6
assign	(resid	70	and	name	HA)	(resid	70	and	name	HB1)	2.9	1.1	1.1
assign	(resid	70	and	name	HA)	(resid	70	and	name	HD1)	3.4	1.6	1.6
assign	(resid	70	and	name	HB1)	(resid	70	and	name	HD2)	2.9	1.1	1.1
assign	(resid	70	and	name	HB2)	(resid	70	and	name	HD1)	2.9	1.1	1.1
assign	(resid	70	and	name	HN)	(resid	70	and	name	HA)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	70	and	name	HB1)	2.9	1.1	1.1
assign	(resid	70	and	name	HN)	(resid	70	and	name	HB2)	2.9	1.1	1.1
assign	(resid	70	and	name	HN)	(resid	70	and	name	HD1)	2.9	1.1	1.1

!GLN

assign	(resid	71	and	name	HB2)	(resid	71	and	name	HA)	2.4	0.6	0.6
assign	(resid	71	and	name	HB1)	(resid	71	and	name	HA)	2.9	1.1	1.1
assign	(resid	71	and	name	HE2*)	(resid	71	and	name	HA)	3.4	1.6	1.6
assign	(resid	71	and	name	HE2*)	(resid	71	and	name	HB1)	3.9	2.1	2.1
assign	(resid	71	and	name	HE2*)	(resid	71	and	name	HB2)	3.9	2.1	2.1
assign	(resid	71	and	name	HE2*)	(resid	71	and	name	HG*)	2.9	1.1	1.1
assign	(resid	71	and	name	HG*)	(resid	71	and	name	HA)	2.9	1.1	1.1
assign	(resid	71	and	name	HG*)	(resid	71	and	name	HB1)	2.4	0.6	0.6
assign	(resid	71	and	name	HN)	(resid	71	and	name	HA)	2.4	0.6	0.6
assign	(resid	71	and	name	HN)	(resid	71	and	name	HB1)	2.4	0.6	0.6
assign	(resid	71	and	name	HN)	(resid	71	and	name	HB2)	2.4	0.6	0.6
assign	(resid	71	and	name	HN)	(resid	71	and	name	HG*)	3.4	1.6	1.6

!GLY

assign	(resid	72	and	name	HN)	(resid	72	and	name	HA*)	2.4	0.6	0.6

!GLY

assign	(resid	73	and	name	HN)	(resid	73	and	name	HA*)	2.4	0.6	0.6

!ASN

assign	(resid	74	and	name	HA)	(resid	74	and	name	HB1)	2.9	1.1	1.1
assign	(resid	74	and	name	HA)	(resid	74	and	name	HB2)	2.9	1.1	1.1
assign	(resid	74	and	name	HD2*)	(resid	74	and	name	HA)	3.9	2.1	2.1
assign	(resid	74	and	name	HD2*)	(resid	74	and	name	HB1)	2.9	1.1	1.1
assign	(resid	74	and	name	HD2*)	(resid	74	and	name	HB2)	2.9	1.1	1.1
assign	(resid	74	and	name	HN)	(resid	74	and	name	HA)	2.9	1.1	1.1
assign	(resid	74	and	name	HN)	(resid	74	and	name	HB1)	3.4	1.6	1.6
assign	(resid	74	and	name	HN)	(resid	74	and	name	HB2)	3.4	1.6	1.6
assign	(resid	74	and	name	HN)	(resid	74	and	name	HD2*)	3.9	2.1	2.1

!ASN

assign	(resid	75	and	name	HB1)	(resid	75	and	name	HA)	3.4	1.6	1.6
assign	(resid	75	and	name	HB2)	(resid	75	and	name	HA)	2.9	1.1	1.1
assign	(resid	75	and	name	HD2*)	(resid	75	and	name	HA)	3.9	2.1	2.1
assign	(resid	75	and	name	HD2*)	(resid	75	and	name	HB1)	2.9	1.1	1.1

assign	(resid	75	and	name	HD2*)	(resid	75	and	name	HB2)	2.9	1.1	1.1
assign	(resid	75	and	name	HD2*)	(resid	75	and	name	HN)	3.9	2.1	2.1
assign	(resid	75	and	name	HN)	(resid	75	and	name	HA)	2.4	0.6	0.6
assign	(resid	75	and	name	HN)	(resid	75	and	name	HB1)	2.9	1.1	1.1
assign	(resid	75	and	name	HN)	(resid	75	and	name	HB2)	3.4	1.6	1.6

!GLU

assign	(resid	76	and	name	HB1)	(resid	76	and	name	HA)	2.4	0.6	0.6
assign	(resid	76	and	name	HB2)	(resid	76	and	name	HA)	2.9	1.1	1.1
assign	(resid	76	and	name	HG*)	(resid	76	and	name	HA)	2.9	1.1	1.1
assign	(resid	76	and	name	HG*)	(resid	76	and	name	HB*)	2.9	1.1	1.1
assign	(resid	76	and	name	HN)	(resid	76	and	name	HA)	2.4	0.6	0.6
assign	(resid	76	and	name	HN)	(resid	76	and	name	HB1)	3.4	1.6	1.6
assign	(resid	76	and	name	HN)	(resid	76	and	name	HB2)	2.9	1.1	1.1
assign	(resid	76	and	name	HN)	(resid	76	and	name	HG*)	2.9	1.1	1.1

!LEU

assign	(resid	77	and	name	HA)	(resid	77	and	name	HB1)	2.4	0.6	0.6
assign	(resid	77	and	name	HA)	(resid	77	and	name	HB2)	2.9	1.1	1.1
assign	(resid	77	and	name	HA)	(resid	77	and	name	HG)	3.9	2.1	2.1
assign	(resid	77	and	name	HD1*)	(resid	77	and	name	HA)	2.4	0.6	0.6
assign	(resid	77	and	name	HD1*)	(resid	77	and	name	HB2)	2.9	1.1	1.1
assign	(resid	77	and	name	HD1*)	(resid	77	and	name	HG)	2.4	0.6	0.6
assign	(resid	77	and	name	HD1*)	(resid	77	and	name	HN)	3.4	1.6	1.6
assign	(resid	77	and	name	HD1*)	(resid	77	and	name	HD2*)	2.4	0.6	0.6
assign	(resid	77	and	name	HD2*)	(resid	77	and	name	HN)	2.9	1.1	1.1
assign	(resid	77	and	name	HD2*)	(resid	77	and	name	HB1)	3.4	1.6	1.6
assign	(resid	77	and	name	HD2*)	(resid	77	and	name	HG)	3.4	1.6	1.6
assign	(resid	77	and	name	HD2*)	(resid	77	and	name	HA)	3.4	1.6	1.6
assign	(resid	77	and	name	HN)	(resid	77	and	name	HA)	3.4	1.6	1.6
assign	(resid	77	and	name	HN)	(resid	77	and	name	HB1)	2.9	1.1	1.1
assign	(resid	77	and	name	HN)	(resid	77	and	name	HB2)	2.4	0.6	0.6
assign	(resid	77	and	name	HN)	(resid	77	and	name	HG)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	78	and	name	HA*)	2.9	1.1	1.1

!THR

assign	(resid	79	and	name	HB)	(resid	79	and	name	HA)	2.4	0.6	0.6
assign	(resid	79	and	name	HG2*)	(resid	79	and	name	HA)	2.9	1.1	1.1
assign	(resid	79	and	name	HG2*)	(resid	79	and	name	HB)	2.4	0.6	0.6
assign	(resid	79	and	name	HN)	(resid	79	and	name	HA)	2.4	0.6	0.6
assign	(resid	79	and	name	HN)	(resid	79	and	name	HB)	2.9	1.1	1.1
assign	(resid	79	and	name	HN)	(resid	79	and	name	HG2*)	3.4	1.6	1.6

!ALA

assign	(resid	80	and	name	HB*)	(resid	80	and	name	HA)	2.4	0.6	0.6
assign	(resid	80	and	name	HN)	(resid	80	and	name	HA)	2.4	0.6	0.6
assign	(resid	80	and	name	HN)	(resid	80	and	name	HB*)	2.4	0.6	0.6

!VAL

assign	(resid	81	and	name	HA)	(resid	81	and	name	HB)	2.9	1.1	1.1
assign	(resid	81	and	name	HG1*)	(resid	81	and	name	HB)	2.4	0.6	0.6
assign	(resid	81	and	name	HG1*)	(resid	81	and	name	HA)	2.9	1.1	1.1
assign	(resid	81	and	name	HG1*)	(resid	81	and	name	HN)	2.9	1.1	1.1
assign	(resid	81	and	name	HG1*)	(resid	81	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	81	and	name	HG2*)	(resid	81	and	name	HB)	2.4	0.6	0.6
assign	(resid	81	and	name	HG2*)	(resid	81	and	name	HA)	2.9	1.1	1.1
assign	(resid	81	and	name	HG2*)	(resid	81	and	name	HN)	2.4	0.6	0.6
assign	(resid	81	and	name	HN)	(resid	81	and	name	HA)	2.9	1.1	1.1
assign	(resid	81	and	name	HN)	(resid	81	and	name	HB)	3.4	1.6	1.6

!GLY

assign	(resid	82	and	name	HN)	(resid	82	and	name	HA*)	2.4	0.6	0.6

!LYS

assign	(resid	83	and	name	HA)	(resid	83	and	name	HB1)	2.4	0.6	0.6
assign	(resid	83	and	name	HA)	(resid	83	and	name	HB2)	2.9	1.1	1.1
assign	(resid	83	and	name	HA)	(resid	83	and	name	HG*)	3.9	2.1	2.1
assign	(resid	83	and	name	HD*)	(resid	83	and	name	HB*)	3.9	2.1	2.1
assign	(resid	83	and	name	HN)	(resid	83	and	name	HA)	2.4	0.6	0.6
assign	(resid	83	and	name	HN)	(resid	83	and	name	HB2)	2.4	0.6	0.6
assign	(resid	83	and	name	HN)	(resid	83	and	name	HB1)	2.9	1.1	1.1
assign	(resid	83	and	name	HN)	(resid	83	and	name	HG*)	3.4	1.6	1.6

!LEU

assign	(resid	84	and	name	HA)	(resid	84	and	name	HB*)	2.9	1.1	1.1
assign	(resid	84	and	name	HN)	(resid	84	and	name	HA)	2.4	0.6	0.6
assign	(resid	84	and	name	HD1*)	(resid	84	and	name	HB*)	3.4	1.6	1.6
assign	(resid	84	and	name	HD1*)	(resid	84	and	name	HG)	2.4	0.6	0.6
assign	(resid	84	and	name	HD1*)	(resid	84	and	name	HA)	3.4	1.6	1.6
assign	(resid	84	and	name	HD1*)	(resid	84	and	name	HN)	3.4	1.6	1.6
assign	(resid	84	and	name	HD2*)	(resid	84	and	name	HB*)	3.4	1.6	1.6
assign	(resid	84	and	name	HD2*)	(resid	84	and	name	HG)	2.4	0.6	0.6
assign	(resid	84	and	name	HD2*)	(resid	84	and	name	HA)	3.4	1.6	1.6
assign	(resid	84	and	name	HD2*)	(resid	84	and	name	HN)	3.4	1.6	1.6
assign	(resid	84	and	name	HN)	(resid	84	and	name	HB*)	2.9	1.1	1.1

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!PHE
assign (resid 85 and name HB1 ) (resid 85 and name HD2 ) 2.9 1.1 1.1
assign (resid 85 and name HB2 ) (resid 85 and name HD1 ) 2.9 1.1 1.1
assign (resid 85 and name HB1 ) (resid 85 and name HA  ) 2.4 0.6 0.6
assign (resid 85 and name HB2 ) (resid 85 and name HA  ) 2.4 0.6 0.6
assign (resid 85 and name HE1 ) (resid 85 and name HB1 ) 3.9 2.1 2.1
assign (resid 85 and name HE2 ) (resid 85 and name HB2 ) 3.9 2.1 2.1
assign (resid 85 and name HN  ) (resid 85 and name HA  ) 2.9 1.1 1.1
assign (resid 85 and name HN  ) (resid 85 and name HB1 ) 2.4 0.6 0.6
assign (resid 85 and name HN  ) (resid 85 and name HB2 ) 2.9 1.1 1.1
assign (resid 85 and name HN  ) (resid 85 and name HD2 ) 2.4 0.6 0.6
assign (resid 85 and name HN  ) (resid 85 and name HE2 ) 3.4 1.6 1.6
assign (resid 85 and name HN  ) (resid 85 and name HZ  ) 3.9 2.1 2.1
!ARG
assign (resid 86 and name HA  ) (resid 86 and name HB* ) 3.4 1.6 1.6
assign (resid 86 and name HA  ) (resid 86 and name HD* ) 3.9 2.1 2.1
assign (resid 86 and name HA  ) (resid 86 and name HG* ) 3.9 2.1 2.1
assign (resid 86 and name HD* ) (resid 86 and name HB* ) 3.9 2.1 2.1
assign (resid 86 and name HE  ) (resid 86 and name HA  ) 3.9 2.1 2.1
assign (resid 86 and name HE  ) (resid 86 and name HG* ) 2.4 0.6 0.6
assign (resid 86 and name HE  ) (resid 86 and name HD* ) 2.4 0.6 0.6
assign (resid 86 and name HE  ) (resid 86 . and name HN  ) 3.4 1.6 1.6
assign (resid 86 and name HG* ) (resid 86 and name HB* ) 2.9 1.1 1.1
assign (resid 86 and name HG* ) (resid 86 and name HD* ) 2.9 1.1 1.1
assign (resid 86 and name HG* ) (resid 86 and name HE  ) 3.9 2.1 2.1
assign (resid 86 and name HN  ) (resid 86 and name HA  ) 2.9 1.1 1.1
assign (resid 86 and name HN  ) (resid 86 and name HB* ) 2.9 1.1 1.1
assign (resid 86 and name HN  ) (resid 86 and name HD* ) 3.9 2.1 2.1
assign (resid 86 and name HN  ) (resid 86 and name HG* ) 3.4 1.6 1.6
!VAL
assign (resid 87 and name HG1* ) (resid 87 and name HA   ) 3.4 1.6 1.6
assign (resid 87 and name HG1* ) (resid 87 and name HN   ) 2.9 1.1 1.1
assign (resid 87 and name HG1* ) (resid 87 and name HB   ) 2.9 1.1 1.1
assign (resid 87 and name HG1* ) (resid 87 and name HG2* ) 2.9 1.1 1.1
assign (resid 87 and name HG2* ) (resid 87 and name HN   ) 3.4 1.6 1.6
assign (resid 87 and name HG2* ) (resid 87 and name HA   ) 2.9 1.1 1.1
assign (resid 87 and name HA   ) (resid 87 and name HB   ) 2.4 0.6 0.6
assign (resid 87 and name HN   ) (resid 87 and name HB   ) 2.4 0.6 0.6
assign (resid 87 and name HN   ) (resid 87 and name HA   ) 2.9 1.1 1.1
!GLY
assign (resid 88 and name HN  ) (resid 88 and name HA* ) 2.9 1.1 1.1
!VAL
assign (resid 89 and name HA   ) (resid 89 and name HB   ) 2.4 0.6 0.6
assign (resid 89 and name HA   ) (resid 89 and name HN   ) 2.9 1.1 1.1
assign (resid 89 and name HG1* ) (resid 89 and name HB   ) 2.9 1.1 1.1
assign (resid 89 and name HG1* ) (resid 89 and name HA   ) 3.4 1.6 1.6
assign (resid 89 and name HG1* ) (resid 89 and name HN   ) 2.9 1.1 1.1
assign (resid 89 and name HG1* ) (resid 89 and name HG2* ) 2.9 1.1 1.1
assign (resid 89 and name HG2* ) (resid 89 and name HB   ) 2.4 0.6 0.6
assign (resid 89 and name HG2* ) (resid 89 and name HA   ) 2.9 1.1 1.1
assign (resid 89 and name HG2* ) (resid 89 and name HN   ) 3.4 1.6 1.6
assign (resid 89 and name HN   ) (resid 89 and name HB   ) 2.4 0.6 0.6
!VAL
assign (resid 90 and name HG1* ) (resid 90 and name HA   ) 3.4 1.6 1.6
assign (resid 90 and name HG1* ) (resid 90 and name HN   ) 3.4 1.6 1.6
assign (resid 90 and name HG1* ) (resid 90 and name HB   ) 2.9 1.1 1.1
assign (resid 90 and name HG1* ) (resid 90 and name HA   ) 2.9 1.1 1.1
assign (resid 90 and name HG2* ) (resid 90 and name HA   ) 2.9 1.1 1.1
assign (resid 90 and name HG2* ) (resid 90 and name HN   ) 2.9 1.1 1.1
assign (resid 90 and name HG2* ) (resid 90 and name HB   ) 2.9 1.1 1.1
assign (resid 90 and name HA   ) (resid 90 and name HB   ) 3.4 1.6 1.6
assign (resid 90 and name HN   ) (resid 90 and name HA   ) 3.4 1.6 1.6
assign (resid 90 and name HN   ) (resid 90 and name HB   ) 2.9 1.1 1.1
!SER
assign (resid 91 and name HB1 ) (resid 91 and name HA  ) 3.4 1.6 1.6
assign (resid 91 and name HB2 ) (resid 91 and name HA  ) 2.9 1.1 1.1
assign (resid 91 and name HN  ) (resid 91 and name HA  ) 3.9 2.1 2.1
assign (resid 91 and name HN  ) (resid 91 and name HB1 ) 2.9 1.1 1.1
assign (resid 91 and name HN  ) (resid 91 and name HB2 ) 2.9 1.1 1.1
!ILE
assign (resid 92 and name HA   ) (resid 92 and name HB   ) 2.9 1.1 1.1
assign (resid 92 and name HA   ) (resid 92 and name HG1* ) 3.9 2.1 2.1
assign (resid 92 and name HD1* ) (resid 92 and name HA   ) 2.9 1.1 1.1
assign (resid 92 and name HD1* ) (resid 92 and name HB   ) 3.4 1.6 1.6
assign (resid 92 and name HD1* ) (resid 92 and name HG1* ) 2.9 1.1 1.1
assign (resid 92 and name HD1* ) (resid 92 and name HG2* ) 2.9 1.1 1.1
assign (resid 92 and name HD1* ) (resid 92 and name HN   ) 3.4 1.6 1.6
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assign (resid 92  and name HG1* ) (resid 92  and name HB  ) 3.9 2.1 2.1
assign (resid 92  and name HG1* ) (resid 92  and name HG2* ) 3.4 1.6 1.6
assign (resid 92  and name HG2* ) (resid 92  and name HA  ) 2.9 1.1 1.1
assign (resid 92  and name HG2* ) (resid 92  and name HB  ) 2.4 0.6 0.6
assign (resid 92  and name HG2* ) (resid 92  and name HN  ) 3.4 1.6 1.6
assign (resid 92  and name HN   ) (resid 92  and name HA  ) 3.4 1.6 1.6
assign (resid 92  and name HN   ) (resid 92  and name HB  ) 2.9 1.1 1.1
!LEU
assign (resid 93  and name HA   ) (resid 93  and name HB2 ) 2.9 1.1 1.1
assign (resid 93  and name HA   ) (resid 93  and name HB1 ) 3.4 1.6 1.6
assign (resid 93  and name HD2* ) (resid 93  and name HA  ) 3.9 2.1 2.1
assign (resid 93  and name HD2* ) (resid 93  and name HB* ) 2.4 0.6 0.6
assign (resid 93  and name HD2* ) (resid 93  and name HG  ) 2.4 0.6 0.6
assign (resid 93  and name HD2* ) (resid 93  and name HD1* ) 2.4 0.6 0.6
assign (resid 93  and name HD2* ) (resid 93  and name HN  ) 3.4 1.6 1.6
assign (resid 93  and name HD1* ) (resid 93  and name HA  ) 2.9 1.1 1.1
assign (resid 93  and name HD1* ) (resid 93  and name HB* ) 2.4 0.6 0.6
assign (resid 93  and name HD1* ) (resid 93  and name HG  ) 2.4 0.6 0.6
assign (resid 93  and name HD1* ) (resid 93  and name HN  ) 3.4 1.6 1.6
assign (resid 93  and name HG   ) (resid 93  and name HA  ) 3.4 1.6 1.6
assign (resid 93  and name HN   ) (resid 93  and name HA  ) 3.4 1.6 1.6
assign (resid 93  and name HN   ) (resid 93  and name HB2 ) 3.4 1.6 1.6
assign (resid 93  and name HN   ) (resid 93  and name HB1 ) 2.9 1.1 1.1
assign (resid 93  and name HN   ) (resid 93  and name HG  ) 3.4 1.6 1.6
!GLU
assign (resid 94  and name HA   ) (resid 94  and name HG* ) 2.9 1.1 1.1
assign (resid 94  and name HB1  ) (resid 94  and name HA  ) 2.4 0.6 0.6
assign (resid 94  and name HB2  ) (resid 94  and name HA  ) 2.9 1.1 1.1
assign (resid 94  and name HB1  ) (resid 94  and name HG* ) 2.9 1.1 1.1
assign (resid 94  and name HB2  ) (resid 94  and name HG* ) 2.9 1.1 1.1
assign (resid 94  and name HN   ) (resid 94  and name HA  ) 2.9 1.1 1.1
assign (resid 94  and name HN   ) (resid 94  and name HB1 ) 2.4 0.6 0.6
assign (resid 94  and name HN   ) (resid 94  and name HB2 ) 2.9 1.1 1.1
!ALA
assign (resid 95  and name HB*  ) (resid 95  and name HA  ) 2.4 0.6 0.6
assign (resid 95  and name HN   ) (resid 95  and name HA  ) 2.9 1.1 1.1
assign (resid 95  and name HN   ) (resid 95  and name HB* ) 2.4 0.6 0.6
!GLY
assign (resid 96  and name HN   ) (resid 96  and name HA* ) 2.4 0.6 0.6
!ASP
assign (resid 97  and name HB1  ) (resid 97  and name HA  ) 2.9 1.1 1.1
assign (resid 97  and name HB2  ) (resid 97  and name HA  ) 2.4 0.6 0.6
assign (resid 97  and name HN   ) (resid 97  and name HA  ) 2.9 1.1 1.1
assign (resid 97  and name HN   ) (resid 97  and name HB1 ) 2.9 1.1 1.1
assign (resid 97  and name HN   ) (resid 97  and name HB2 ) 2.4 0.6 0.6
!SER
assign (resid 98  and name HB*  ) (resid 98  and name HA  ) 2.9 1.1 1.1
assign (resid 98  and name HN   ) (resid 98  and name HA  ) 2.4 0.6 0.6
assign (resid 98  and name HN   ) (resid 98  and name HB* ) 2.4 0.6 0.6
!ASP
assign (resid 99  and name HB1  ) (resid 99  and name HA  ) 2.4 0.6 0.6
assign (resid 99  and name HB2  ) (resid 99  and name HA  ) 2.4 0.6 0.6
assign (resid 99  and name HN   ) (resid 99  and name HA  ) 3.4 1.6 1.6
assign (resid 99  and name HN   ) (resid 99  and name HB2 ) 2.9 1.1 1.1
assign (resid 99  and name HN   ) (resid 99  and name HB1 ) 3.4 1.6 1.6
!ILE
assign (resid 100 and name HA   ) (resid 100 and name HB  ) 2.9 1.1 1.1
assign (resid 100 and name HA   ) (resid 100 and name HG1* ) 3.4 1.6 1.6
assign (resid 100 and name HD1* ) (resid 100 and name HA  ) 3.4 1.6 1.6
assign (resid 100 and name HD1* ) (resid 100 and name HB  ) 2.9 1.1 1.1
assign (resid 100 and name HG1* ) (resid 100 and name HB  ) 2.9 1.1 1.1
assign (resid 100 and name HG1* ) (resid 100 and name HD1* ) 2.4 0.6 0.6
assign (resid 100 and name HG2* ) (resid 100 and name HA  ) 2.9 1.1 1.1
assign (resid 100 and name HG2* ) (resid 100 and name HB  ) 2.4 0.6 0.6
assign (resid 100 and name HG2* ) (resid 100 and name HD1* ) 2.9 1.1 1.1
assign (resid 100 and name HG2* ) (resid 100 and name HG1* ) 2.9 1.1 1.1
assign (resid 100 and name HN   ) (resid 100 and name HA  ) 2.9 1.1 1.1
assign (resid 100 and name HN   ) (resid 100 and name HB  ) 3.9 2.1 2.1
assign (resid 100 and name HN   ) (resid 100 and name HD1* ) 2.9 1.1 1.1
assign (resid 100 and name HN   ) (resid 100 and name HG1* ) 2.9 1.1 1.1
assign (resid 100 and name HN   ) (resid 100 and name HG2* ) 2.9 1.1 1.1
!LEU
assign (resid 101 and name HA   ) (resid 101 and name HN  ) 2.9 1.1 1.1
assign (resid 101 and name HB*  ) (resid 101 and name HA  ) 2.9 1.1 1.1
assign (resid 101 and name HD1* ) (resid 101 and name HA  ) 3.4 1.6 1.6
assign (resid 101 and name HD1* ) (resid 101 and name HB* ) 2.4 0.6 0.6
assign (resid 101 and name HD1* ) (resid 101 and name HG  ) 2.4 0.6 0.6
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assign	(resid	101	and	name	HD1*)	(resid	101	and	name	HN)	2.9	1.1	1.1
assign	(resid	101	and	name	HD1*)	(resid	101	and	name	HD2*)	2.4	0.6	0.6
assign	(resid	101	and	name	HD2*)	(resid	101	and	name	HA)	3.4	1.6	1.6
assign	(resid	101	and	name	HD2*)	(resid	101	and	name	HB*)	2.4	0.6	0.6
assign	(resid	101	and	name	HD2*)	(resid	101	and	name	HG)	2.4	0.6	0.6
assign	(resid	101	and	name	HD2*)	(resid	101	and	name	HN)	2.9	1.1	1.1
assign	(resid	101	and	name	HG)	(resid	101	and	name	HA)	2.9	1.1	1.1
assign	(resid	101	and	name	HG)	(resid	101	and	name	HB*)	2.4	0.6	0.6
assign	(resid	101	and	name	HN)	(resid	101	and	name	HB*)	2.9	1.1	1.1
assign	(resid	101	and	name	HN)	(resid	101	and	name	HG)	2.9	1.1	1.1

!THR

assign	(resid	102	and	name	HA)	(resid	102	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	102	and	name	HB)	(resid	102	and	name	HA)	2.4	0.6	0.6
assign	(resid	102	and	name	HB)	(resid	102	and	name	HN)	3.4	1.6	1.6
assign	(resid	102	and	name	HG2*)	(resid	102	and	name	HB)	2.9	1.1	1.1
assign	(resid	102	and	name	HN)	(resid	102	and	name	HA)	2.4	0.6	0.6
assign	(resid	102	and	name	HN)	(resid	102	and	name	HG2*)	2.4	0.6	0.6

!THR

assign	(resid	103	and	name	HA)	(resid	103	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	103	and	name	HB)	(resid	103	and	name	HA)	2.9	1.1	1.1
assign	(resid	103	and	name	HG2*)	(resid	103	and	name	HB)	2.4	0.6	0.6
assign	(resid	103	and	name	HN)	(resid	103	and	name	HA)	2.4	0.6	0.6
assign	(resid	103	and	name	HN)	(resid	103	and	name	HB)	2.9	1.1	1.1
assign	(resid	103	and	name	HN)	(resid	103	and	name	HG2*)	2.9	1.1	1.1

!LEU

assign	(resid	104	and	name	HB*)	(resid	104	and	name	HA)	2.9	1.1	1.1
assign	(resid	104	and	name	HB*)	(resid	104	and	name	HD*)	2.9	1.1	1.1
assign	(resid	104	and	name	HB*)	(resid	104	and	name	HG)	3.9	2.1	2.1
assign	(resid	104	and	name	HB*)	(resid	104	and	name	HN)	2.9	1.1	1.1
assign	(resid	104	and	name	HD*)	(resid	104	and	name	HA)	3.4	1.6	1.6
assign	(resid	104	and	name	HD*)	(resid	104	and	name	HN)	3.4	1.6	1.6
assign	(resid	104	and	name	HG)	(resid	104	and	name	HA)	3.9	2.1	2.1
assign	(resid	104	and	name	HG)	(resid	104	and	name	HD*)	2.4	0.6	0.6
assign	(resid	104	and	name	HG)	(resid	104	and	name	HN)	3.4	1.6	1.6
assign	(resid	104	and	name	HA)	(resid	104	and	name	HN)	2.9	1.1	1.1
assign	(resid	104	and	name	HN)	(resid	104	and	name	HA)	2.4	0.6	0.6

!ALA

assign	(resid	105	and	name	HB*)	(resid	105	and	name	HA)	2.4	0.6	0.6
assign	(resid	105	and	name	HN)	(resid	105	and	name	HB*)	2.4	0.6	0.6
assign	(resid	105	and	name	HN)	(resid	105	and	name	HA)	2.4	0.6	0.6

!protein inter-residue restraints
!ALA2

assign	(resid	2	and	name	HA)	(resid	3	and	name	HD*)	2.9	1.1	1.1
assign	(resid	2	and	name	HB*)	(resid	3	and	name	HG*)	3.4	1.6	1.6
assign	(resid	2	and	name	HB*)	(resid	3	and	name	HD*)	3.4	1.6	1.6

!VAL4

assign	(resid	4	and	name	HA)	(resid	5	and	name	HN)	2.9	1.1	1.1
assign	(resid	4	and	name	HB)	(resid	5	and	name	HN)	2.9	1.1	1.1
assign	(resid	4	and	name	HG1*)	(resid	5	and	name	HN)	3.4	1.6	1.6
assign	(resid	4	and	name	HG2*)	(resid	5	and	name	HN)	3.4	1.6	1.6
assign	(resid	4	and	name	HN)	(resid	3	and	name	HA)	2.4	0.6	0.6
assign	(resid	4	and	name	HN)	(resid	3	and	name	HD*)	3.4	1.6	1.6
assign	(resid	4	and	name	HN)	(resid	5	and	name	HN)	3.4	1.6	1.6

!LYS5

assign	(resid	5	and	name	HB1)	(resid	4	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	5	and	name	HB1)	(resid	4	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	5	and	name	HD*)	(resid	3	and	name	HA)	3.9	2.1	2.1
assign	(resid	5	and	name	HE*)	(resid	3	and	name	HB2)	2.4	0.6	0.6
assign	(resid	5	and	name	HE*)	(resid	4	and	name	HB)	3.4	1.6	1.6
assign	(resid	5	and	name	HG*)	(resid	6	and	name	HA)	2.9	1.1	1.1
assign	(resid	5	and	name	HG*)	(resid	8	and	name	HG*)	3.9	2.1	2.1
assign	(resid	5	and	name	HN)	(resid	6	and	name	HN)	2.9	1.1	1.1

!SER6

assign	(resid	6	and	name	HB*)	(resid	2	and	name	HA)	2.9	1.1	1.1
assign	(resid	6	and	name	HB*)	(resid	5	and	name	HB*)	3.9	2.1	2.1
assign	(resid	6	and	name	HB*)	(resid	7	and	name	HN)	3.4	1.6	1.6
assign	(resid	6	and	name	HN)	(resid	4	and	name	HG*)	3.9	2.1	2.1
assign	(resid	6	and	name	HN)	(resid	5	and	name	HA)	2.9	1.1	1.1
assign	(resid	6	and	name	HN)	(resid	5	and	name	HB*)	3.4	1.6	1.6
assign	(resid	6	and	name	HN)	(resid	5	and	name	HG*)	3.9	2.1	2.1
assign	(resid	6	and	name	HN)	(resid	7	and	name	HN)	3.4	1.6	1.6

!GLN7

assign	(resid	7	and	name	HA)	(resid	8	and	name	HN)	2.9	1.1	1.1
assign	(resid	7	and	name	HA)	(resid	10	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	7	and	name	HA)	(resid	10	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	7	and	name	HB*)	(resid	6	and	name	HB*)	3.9	2.1	2.1

assign	(resid	7	and	name	HB*)	(resid	8	and	name	HN)	3.4	1.6	1.6
assign	(resid	7	and	name	HE2*)	(resid	9	and	name	HN)	3.9	2.1	2.1
assign	(resid	7	and	name	HE2*)	(resid	10	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	7	and	name	HE2*)	(resid	10	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	7	and	name	HE2*)	(resid	70	and	name	HD1)	3.4	1.6	1.6
assign	(resid	7	and	name	HE2*)	(resid	70	and	name	HE1)	3.4	1.6	1.6
assign	(resid	7	and	name	HN)	(resid	6	and	name	HA)	2.9	1.1	1.1
assign	(resid	7	and	name	HN)	(resid	8	and	name	HN)	2.9	1.1	1.1

!GLU8

assign	(resid	8	and	name	HA)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	8	and	name	HA)	(resid	11	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	8	and	name	HG*)	(resid	4	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	8	and	name	HG*)	(resid	5	and	name	HG*)	3.9	2.1	2.1
assign	(resid	8	and	name	HG*)	(resid	9	and	name	HB*)	3.9	2.1	2.1
assign	(resid	8	and	name	HN)	(resid	9	and	name	HB*)	3.9	2.1	2.1
assign	(resid	8	and	name	HN)	(resid	9	and	name	HN)	2.9	1.1	1.1

!SER9

assign	(resid	9	and	name	HA)	(resid	12	and	name	HB1)	2.9	1.1	1.1
assign	(resid	9	and	name	HA)	(resid	12	and	name	HB2)	3.4	1.6	1.6
assign	(resid	9	and	name	HA)	(resid	12	and	name	HG*)	3.9	2.1	2.1
assign	(resid	9	and	name	HB*)	(resid	10	and	name	HN)	3.4	1.6	1.6
assign	(resid	9	and	name	HN)	(resid	8	and	name	HA)	2.9	1.1	1.1
assign	(resid	9	and	name	HN)	(resid	8	and	name	HG*)	3.4	1.6	1.6
assign	(resid	9	and	name	HN)	(resid	8	and	name	HB*)	2.9	1.1	1.1
assign	(resid	9	and	name	HN)	(resid	10	and	name	HN)	2.9	1.1	1.1
assign	(resid	9	and	name	HN)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	9	and	name	HN)	(resid	8	and	name	HG*)	3.9	2.1	2.1
assign	(resid	9	and	name	HN)	(resid	8	and	name	HB*)	2.9	1.1	1.1
assign	(resid	9	and	name	HN)	(resid	11	and	name	HN)	3.4	1.6	1.6
assign	(resid	9	and	name	HN)	(resid	12	and	name	HN)	3.4	1.6	1.6

!ILE10

assign	(resid	10	and	name	HA)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	10	and	name	HA)	(resid	13	and	name	HB*)	2.9	1.1	1.1
assign	(resid	10	and	name	HA)	(resid	14	and	name	HB1)	3.9	2.1	2.1
assign	(resid	10	and	name	HA)	(resid	14	and	name	HB2)	2.9	1.1	1.1
assign	(resid	10	and	name	HA)	(resid	104	and	name	HD*)	3.9	2.1	2.1
assign	(resid	10	and	name	HB)	(resid	11	and	name	HN)	2.9	1.1	1.1
assign	(resid	10	and	name	HB)	(resid	104	and	name	HD*)	3.9	2.1	2.1
assign	(resid	10	and	name	HD1*)	(resid	7	and	name	HA)	3.9	2.1	2.1
assign	(resid	10	and	name	HD1*)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	10	and	name	HD1*)	(resid	14	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	10	and	name	HD1*)	(resid	43	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	10	and	name	HD1*)	(resid	68	and	name	HD2)	3.4	1.6	1.6
assign	(resid	10	and	name	HD1*)	(resid	68	and	name	HE2)	3.4	1.6	1.6
assign	(resid	10	and	name	HD1*)	(resid	70	and	name	HE1)	2.9	1.1	1.1
assign	(resid	10	and	name	HD1*)	(resid	14	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	10	and	name	HG2*)	(resid	9	and	name	HA)	3.9	2.1	2.1
assign	(resid	10	and	name	HG2*)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	10	and	name	HG2*)	(resid	11	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	10	and	name	HG2*)	(resid	11	and	name	HN)	2.9	1.1	1.1
assign	(resid	10	and	name	HG2*)	(resid	12	and	name	HN)	3.9	2.1	2.1
assign	(resid	10	and	name	HG2*)	(resid	43	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	10	and	name	HG2*)	(resid	68	and	name	HE2)	3.4	1.6	1.6
assign	(resid	10	and	name	HG2*)	(resid	68	and	name	HD2)	3.4	1.6	1.6
assign	(resid	10	and	name	HG2*)	(resid	70	and	name	HE1)	2.9	1.1	1.1
assign	(resid	10	and	name	HG2*)	(resid	104	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	10	and	name	HN)	(resid	9	and	name	HA)	2.9	1.1	1.1
assign	(resid	10	and	name	HN)	(resid	8	and	name	HA)	3.4	1.6	1.6
assign	(resid	10	and	name	HN)	(resid	11	and	name	HB*)	3.9	2.1	2.1
assign	(resid	10	and	name	HN)	(resid	11	and	name	HN)	2.9	1.1	1.1
assign	(resid	10	and	name	HN)	(resid	12	and	name	HN)	3.9	2.1	2.1

!ASN11

assign	(resid	11	and	name	HA)	(resid	14	and	name	HB1)	2.9	1.1	1.1
assign	(resid	11	and	name	HA)	(resid	14	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	11	and	name	HA)	(resid	14	and	name	HN)	2.9	1.1	1.1
assign	(resid	11	and	name	HD2*)	(resid	9	and	name	HB*)	3.9	2.1	2.1
assign	(resid	11	and	name	HD2*)	(resid	79	and	name	HB)	3.9	2.1	2.1
assign	(resid	11	and	name	HD2*)	(resid	70	and	name	HZ)	3.9	2.1	2.1
assign	(resid	11	and	name	HN)	(resid	9	and	name	HB*)	3.4	1.6	1.6
assign	(resid	11	and	name	HN)	(resid	9	and	name	HA)	2.9	1.1	1.1
assign	(resid	11	and	name	HN)	(resid	9	and	name	HN)	3.9	2.1	2.1
assign	(resid	11	and	name	HN)	(resid	10	and	name	HA)	3.4	1.6	1.6
assign	(resid	11	and	name	HN)	(resid	10	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	11	and	name	HN)	(resid	10	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	11	and	name	HN)	(resid	7	and	name	HB*)	3.4	1.6	1.6
assign	(resid	11	and	name	HN)	(resid	7	and	name	HG*)	3.4	1.6	1.6
assign	(resid	11	and	name	HN)	(resid	12	and	name	HN)	2.4	0.6	0.6

assign	(resid	11	and	name	HN)	(resid	13	and	name	HN)	3.9	2.1	2.1
assign	(resid	11	and	name	HN)	(resid	70	and	name	HZ)	3.9	2.1	2.1

!GLN12

assign	(resid	12	and	name	HA)	(resid	15	and	name	HB*)	2.9	1.1	1.1
assign	(resid	12	and	name	HA)	(resid	15	and	name	HN)	2.9	1.1	1.1
assign	(resid	12	and	name	HB1)	(resid	13	and	name	HN)	3.4	1.6	1.6
assign	(resid	12	and	name	HB2)	(resid	13	and	name	HN)	3.4	1.6	1.6
assign	(resid	12	and	name	HB1)	(resid	15	and	name	HB*)	3.9	2.1	2.1
assign	(resid	12	and	name	HB1)	(resid	15	and	name	HN)	3.9	2.1	2.1
assign	(resid	12	and	name	HG*)	(resid	13	and	name	HN)	3.4	1.6	1.6
assign	(resid	12	and	name	HG*)	(resid	15	and	name	HB*)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	9	and	name	HA)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	9	and	name	HB*)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	10	and	name	HA)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	10	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	10	and	name	HB*)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	11	and	name	HA)	3.4	1.6	1.6
assign	(resid	12	and	name	HN)	(resid	11	and	name	HB*)	2.9	1.1	1.1
assign	(resid	12	and	name	HN)	(resid	13	and	name	HG*)	3.9	2.1	2.1
assign	(resid	12	and	name	HN)	(resid	13	and	name	HN)	2.9	1.1	1.1

!LYS13

assign	(resid	13	and	name	HA)	(resid	16	and	name	HB2)	2.9	1.1	1.1
assign	(resid	13	and	name	HA)	(resid	16	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	13	and	name	HA)	(resid	16	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	13	and	name	HA)	(resid	16	and	name	HG)	3.9	2.1	2.1
assign	(resid	13	and	name	HN)	(resid	10	and	name	HA)	2.9	1.1	1.1
assign	(resid	13	and	name	HN)	(resid	11	and	name	HA)	3.9	2.1	2.1
assign	(resid	13	and	name	HN)	(resid	12	and	name	HA)	2.9	1.1	1.1
assign	(resid	13	and	name	HN)	(resid	14	and	name	HN)	2.9	1.1	1.1

!LEU14

assign	(resid	14	and	name	HA)	(resid	17	and	name	HB)	2.9	1.1	1.1
assign	(resid	14	and	name	HA)	(resid	17	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	14	and	name	HA)	(resid	17	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	14	and	name	HB1)	(resid	15	and	name	HN)	2.9	1.1	1.1
assign	(resid	14	and	name	HB2)	(resid	15	and	name	HN)	3.4	1.6	1.6
assign	(resid	14	and	name	HD2*)	(resid	11	and	name	HA)	3.9	2.1	2.1
assign	(resid	14	and	name	HD2*)	(resid	15	and	name	HN)	3.4	1.6	1.6
assign	(resid	14	and	name	HD2*)	(resid	43	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	14	and	name	HD2*)	(resid	77	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	14	and	name	HD1*)	(resid	17	and	name	HN)	3.9	2.1	2.1
assign	(resid	14	and	name	HD1*)	(resid	43	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	14	and	name	HD1*)	(resid	77	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	14	and	name	HN)	(resid	10	and	name	HA)	2.9	1.1	1.1
assign	(resid	14	and	name	HN)	(resid	10	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	14	and	name	HN)	(resid	10	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	14	and	name	HN)	(resid	13	and	name	HA)	3.4	1.6	1.6
assign	(resid	14	and	name	HN)	(resid	13	and	name	HB*)	2.9	1.1	1.1
assign	(resid	14	and	name	HN)	(resid	13	and	name	HG*)	3.4	1.6	1.6
assign	(resid	14	and	name	HN)	(resid	15	and	name	HN)	2.9	1.1	1.1

!ALA15

assign	(resid	15	and	name	HA)	(resid	12	and	name	HA)	3.4	1.6	1.6
assign	(resid	15	and	name	HA)	(resid	16	and	name	HA)	3.9	2.1	2.1
assign	(resid	15	and	name	HA)	(resid	17	and	name	HN)	3.9	2.1	2.1
assign	(resid	15	and	name	HA)	(resid	18	and	name	HA)	3.9	2.1	2.1
assign	(resid	15	and	name	HA)	(resid	18	and	name	HB)	2.9	1.1	1.1
assign	(resid	15	and	name	HA)	(resid	18	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	15	and	name	HA)	(resid	18	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	15	and	name	HB*)	(resid	11	and	name	HA)	3.9	2.1	2.1
assign	(resid	15	and	name	HB*)	(resid	14	and	name	HN)	3.9	2.1	2.1
assign	(resid	15	and	name	HB*)	(resid	16	and	name	HN)	2.9	1.1	1.1
assign	(resid	15	and	name	HB*)	(resid	18	and	name	HN)	3.4	1.6	1.6
assign	(resid	15	and	name	HB*)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	15	and	name	HN)	(resid	12	and	name	HG*)	3.9	2.1	2.1
assign	(resid	15	and	name	HN)	(resid	14	and	name	HA)	3.4	1.6	1.6
assign	(resid	15	and	name	HN)	(resid	16	and	name	HN)	2.9	1.1	1.1
assign	(resid	15	and	name	HN)	(resid	18	and	name	HN)	3.4	1.6	1.6
assign	(resid	15	and	name	HN)	(resid	18	and	name	HG1*)	3.9	2.1	2.1

!LEU16

assign	(resid	16	and	name	HA)	(resid	17	and	name	HA)	3.9	2.1	2.1
assign	(resid	16	and	name	HA)	(resid	19	and	name	HB*)	2.9	1.1	1.1
assign	(resid	16	and	name	HA)	(resid	19	and	name	HG*)	3.9	2.1	2.1
assign	(resid	16	and	name	HB2)	(resid	17	and	name	HN)	3.4	1.6	1.6
assign	(resid	16	and	name	HB1)	(resid	17	and	name	HN)	2.9	1.1	1.1
assign	(resid	16	and	name	HB1)	(resid	17	and	name	HN)	3.4	1.6	1.6
assign	(resid	16	and	name	HD1*)	(resid	17	and	name	HN)	3.9	2.1	2.1
assign	(resid	16	and	name	HD1*)	(resid	19	and	name	HD*)	3.4	1.6	1.6
assign	(resid	16	and	name	HD1*)	(resid	19	and	name	HE*)	3.4	1.6	1.6

assign	(resid	16	and	name	HD1*)	(resid	97	and	name	HB1)	3.4	1.6	1.6
assign	(resid	16	and	name	HD1*)	(resid	99	and	name	HN)	3.9	2.1	2.1
assign	(resid	16	and	name	HD2*)	(resid	17	and	name	HN)	3.4	1.6	1.6
assign	(resid	16	and	name	HD2*)	(resid	19	and	name	HD*)	3.4	1.6	1.6
assign	(resid	16	and	name	HD2*)	(resid	19	and	name	HE*)	3.4	1.6	1.6
assign	(resid	16	and	name	HD2*)	(resid	97	and	name	HB1)	3.4	1.6	1.6
assign	(resid	16	and	name	HD2*)	(resid	97	and	name	HB2)	3.4	1.6	1.6
assign	(resid	16	and	name	HD2*)	(resid	99	and	name	HN)	3.9	2.1	2.1
assign	(resid	16	and	name	HG)	(resid	17	and	name	HA)	3.9	2.1	2.1
assign	(resid	16	and	name	HG)	(resid	98	and	name	HN)	3.9	2.1	2.1
assign	(resid	16	and	name	HN)	(resid	13	and	name	HA)	2.9	1.1	1.1
assign	(resid	16	and	name	HN)	(resid	14	and	name	HA)	3.4	1.6	1.6
assign	(resid	16	and	name	HN)	(resid	14	and	name	HN)	3.9	2.1	2.1
assign	(resid	16	and	name	HN)	(resid	15	and	name	HA)	2.9	1.1	1.1
assign	(resid	16	and	name	HN)	(resid	17	and	name	HA)	3.9	2.1	2.1
assign	(resid	16	and	name	HN)	(resid	17	and	name	HN)	2.9	1.1	1

!VAL17

assign	(resid	17	and	name	HA)	(resid	19	and	name	HG*)	3.9	2.1	2.1
assign	(resid	17	and	name	HA)	(resid	21	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HA)	(resid	92	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	17	and	name	HA)	(resid	96	and	name	HN)	2.9	1.1	1.1
assign	(resid	17	and	name	HA)	(resid	97	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HG1*)	(resid	18	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	17	and	name	HG1*)	(resid	92	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	17	and	name	HG1*)	(resid	21	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HG1*)	(resid	23	and	name	HB1)	3.4	1.6	1.6
assign	(resid	17	and	name	HG1*)	(resid	23	and	name	HD1)	3.9	2.1	2.1
assign	(resid	17	and	name	HG1*)	(resid	94	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HG1*)	(resid	95	and	name	HA)	3.4	1.6	1.6
assign	(resid	17	and	name	HG1*)	(resid	96	and	name	HN)	3.4	1.6	1.6
assign	(resid	17	and	name	HG2*)	(resid	21	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HG2*)	(resid	95	and	name	HA)	3.4	1.6	1.6
assign	(resid	17	and	name	HG2*)	(resid	96	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HG2*)	(resid	98	and	name	HN)	3.4	1.6	1.6
assign	(resid	17	and	name	HG2*)	(resid	100	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	17	and	name	HN)	(resid	14	and	name	HA)	2.9	1.1	1.1
assign	(resid	17	and	name	HN)	(resid	16	and	name	HA)	2.9	1.1	1.1
assign	(resid	17	and	name	HN)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	17	and	name	HN)	(resid	18	and	name	HN)	2.9	1.1	1.1
assign	(resid	17	and	name	HN)	(resid	19	and	name	HN)	3.9	2.1	2.1
assign	(resid	17	and	name	HN)	(resid	100	and	name	HD1*)	3.9	2.1	2.1

!ILE18

assign	(resid	18	and	name	HA)	(resid	23	and	name	HD1)	2.9	1.1	1.1
assign	(resid	18	and	name	HA)	(resid	23	and	name	HE1)	2.9	1.1	1.1
assign	(resid	18	and	name	HB)	(resid	19	and	name	HN)	2.9	1.1	1.1
assign	(resid	18	and	name	HD1*)	(resid	14	and	name	HA)	3.9	2.1	2.1
assign	(resid	18	and	name	HD1*)	(resid	19	and	name	HN)	3.9	2.1	2.1
assign	(resid	18	and	name	HD1*)	(resid	23	and	name	HD2)	2.9	1.1	1.1
assign	(resid	18	and	name	HD1*)	(resid	23	and	name	HE2)	3.4	1.6	1.6
assign	(resid	18	and	name	HD1*)	(resid	81	and	name	HA)	2.9	1.1	1.1
assign	(resid	18	and	name	HD1*)	(resid	81	and	name	HB)	2.9	1.1	1.1
assign	(resid	18	and	name	HG1*)	(resid	15	and	name	HA)	3.9	2.1	2.1
assign	(resid	18	and	name	HG1*)	(resid	23	and	name	HE1)	3.4	1.6	1.6
assign	(resid	18	and	name	HG2*)	(resid	19	and	name	HN)	2.9	1.1	1.1
assign	(resid	18	and	name	HG2*)	(resid	23	and	name	HD1)	3.4	1.6	1.6
assign	(resid	18	and	name	HG2*)	(resid	23	and	name	HE1)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	14	and	name	HA)	3.9	2.1	2.1
assign	(resid	18	and	name	HN)	(resid	15	and	name	HA)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	16	and	name	HA)	3.9	2.1	2.1
assign	(resid	18	and	name	HN)	(resid	17	and	name	HA)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	17	and	name	HB)	3.9	2.1	2.1
assign	(resid	18	and	name	HN)	(resid	17	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	19	and	name	HN)	2.9	1.1	1.1
assign	(resid	18	and	name	HN)	(resid	21	and	name	HN)	3.4	1.6	1.6
assign	(resid	18	and	name	HN)	(resid	23	and	name	HE1)	3.9	2.1	2.1

!LYS19

assign	(resid	19	and	name	HA)	(resid	20	and	name	HN)	2.9	1.1	1.1
assign	(resid	19	and	name	HG*)	(resid	16	and	name	HG)	3.9	2.1	2.1
assign	(resid	19	and	name	HB*)	(resid	18	and	name	HN)	3.9	2.1	2.1
assign	(resid	19	and	name	HB*)	(resid	20	and	name	HN)	3.4	1.6	1.6
assign	(resid	19	and	name	HN)	(resid	15	and	name	HA)	3.4	1.6	1.6
assign	(resid	19	and	name	HN)	(resid	16	and	name	HA)	2.9	1.1	1.1
assign	(resid	19	and	name	HN)	(resid	16	and	name	HN)	3.9	2.1	2.1
assign	(resid	19	and	name	HN)	(resid	16	and	name	HD*)	3.9	2.1	2.1
assign	(resid	19	and	name	HN)	(resid	18	and	name	HA)	2.9	1.1	1.1
assign	(resid	19	and	name	HN)	(resid	18	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	19	and	name	HN)	(resid	20	and	name	HA)	3.9	2.1	2.1

assign	(resid	19	and	name	HN)	(resid	20	and	name	HB1)	3.9	2.1	2.1
assign	(resid	19	and	name	HN)	(resid	20	and	name	HB2)	3.9	2.1	2.1
assign	(resid	19	and	name	HN)	(resid	20	and	name	HN)	2.9	1.1	1.1
assign	(resid	19	and	name	HN)	(resid	21	and	name	HN)	3.9	2.1	2.1

!SER20

assign	(resid	20	and	name	HA)	(resid	19	and	name	HG*)	3.9	2.1	2.1
assign	(resid	20	and	name	HN)	(resid	17	and	name	HA)	2.9	1.1	1
assign	(resid	20	and	name	HN)	(resid	19	and	name	HD*)	3.9	2.1	2.1
assign	(resid	20	and	name	HN)	(resid	19	and	name	HG*)	2.9	1.1	1.1
assign	(resid	20	and	name	HN)	(resid	21	and	name	HN)	2.4	0.6	0.6
assign	(resid	20	and	name	HN)	(resid	97	and	name	HN)	3.9	2.1	2.1

!GLY21

assign	(resid	21	and	name	HN)	(resid	94	and	name	HG*)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	18	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	19	and	name	HA)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	19	and	name	HB*)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	19	and	name	HG*)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	20	and	name	HA)	3.4	1.6	1.6
assign	(resid	21	and	name	HN)	(resid	20	and	name	HB2)	3.9	2.1	2.1
assign	(resid	21	and	name	HN)	(resid	22	and	name	HN)	3.4	1.6	1.6
assign	(resid	21	and	name	HN)	(resid	96	and	name	HA#)	2.4	0.6	0.6
assign	(resid	21	and	name	HN)	(resid	96	and	name	HN)	3.9	2.1	2.1

!LYS22

assign	(resid	22	and	name	HB1)	(resid	23	and	name	HN)	2.9	1.1	1.1
assign	(resid	22	and	name	HB2)	(resid	93	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	22	and	name	HB2)	(resid	94	and	name	HG*)	3.4	1.6	1.6
assign	(resid	22	and	name	HB2)	(resid	94	and	name	HN)	2.9	1.1	1.1
assign	(resid	22	and	name	HG*)	(resid	93	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	22	and	name	HG*)	(resid	94	and	name	HG*)	3.9	2.1	2.1
assign	(resid	22	and	name	HN)	(resid	18	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	22	and	name	HN)	(resid	21	and	name	HA*)	2.4	0.6	0.6
assign	(resid	22	and	name	HN)	(resid	23	and	name	HN)	3.9	2.1	2.1
assign	(resid	22	and	name	HN)	(resid	94	and	name	HB1)	3.4	1.6	1.6
assign	(resid	22	and	name	HN)	(resid	94	and	name	HB2)	3.4	1.6	1.6
assign	(resid	22	and	name	HN)	(resid	94	and	name	HN)	2.9	1.1	1.1
assign	(resid	22	and	name	HN)	(resid	96	and	name	HN)	3.9	2.1	2.1

!TYR23

assign	(resid	23	and	name	HA)	(resid	92	and	name	HA)	2.4	0.6	0.6
assign	(resid	23	and	name	HA)	(resid	92	and	name	HB)	3.9	2.1	2.1
assign	(resid	23	and	name	HA)	(resid	92	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	23	and	name	HA)	(resid	92	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	23	and	name	HA)	(resid	93	and	name	HN)	2.9	1.1	1.1
assign	(resid	23	and	name	HD1)	(resid	22	and	name	HA)	3.4	1.6	1.6
assign	(resid	23	and	name	HD2)	(resid	24	and	name	HA)	3.9	2.1	2.1
assign	(resid	23	and	name	HD2)	(resid	25	and	name	HG)	2.4	0.6	0.6
assign	(resid	23	and	name	HE1)	(resid	22	and	name	HA)	3.9	2.1	2.1
assign	(resid	23	and	name	HE2)	(resid	24	and	name	HA)	3.9	2.1	2.1
assign	(resid	23	and	name	HE2)	(resid	25	and	name	HG)	2.9	1.1	1.1
assign	(resid	23	and	name	HN)	(resid	18	and	name	HA)	3.4	1.6	1.6
assign	(resid	23	and	name	HN)	(resid	22	and	name	HA)	2.4	0.6	0.6
assign	(resid	23	and	name	HN)	(resid	22	and	name	HB*)	3.4	1.6	1.6
assign	(resid	23	and	name	HN)	(resid	22	and	name	HG*)	3.4	1.6	1.6
assign	(resid	23	and	name	HN)	(resid	24	and	name	HN)	3.4	1.6	1.6

!THR24

assign	(resid	24	and	name	HG2*)	(resid	91	and	name	HB1)	2.9	1.1	1.1
assign	(resid	24	and	name	HG2*)	(resid	91	and	name	HN)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	23	and	name	HA)	2.4	0.6	0.6
assign	(resid	24	and	name	HN)	(resid	23	and	name	HB*)	2.9	1.1	1.1
assign	(resid	24	and	name	HN)	(resid	23	and	name	HD2)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	23	and	name	HE2)	3.9	2.1	2.1
assign	(resid	24	and	name	HN)	(resid	25	and	name	HN)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	34	and	name	HG)	3.9	2.1	2.1
assign	(resid	24	and	name	HN)	(resid	90	and	name	HA)	3.9	2.1	2.1
assign	(resid	24	and	name	HN)	(resid	91	and	name	HB1)	2.9	1.1	1.1
assign	(resid	24	and	name	HN)	(resid	91	and	name	HB2)	3.9	2.1	2.1
assign	(resid	24	and	name	HN)	(resid	91	and	name	HN)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	90	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	92	and	name	HA)	3.4	1.6	1.6
assign	(resid	24	and	name	HN)	(resid	92	and	name	HD1*)	2.9	1.1	1.1

!LEU25

assign	(resid	25	and	name	HA)	(resid	90	and	name	HA)	2.4	0.6	0.6
assign	(resid	25	and	name	HD1*)	(resid	23	and	name	HB2)	3.4	1.6	1.6
assign	(resid	25	and	name	HD1*)	(resid	23	and	name	HD2)	2.9	1.1	1.1
assign	(resid	25	and	name	HD1*)	(resid	23	and	name	HE*)	3.4	1.6	1.6
assign	(resid	25	and	name	HD2*)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	25	and	name	HD2*)	(resid	23	and	name	HD2)	2.9	1.1	1.1
assign	(resid	25	and	name	HD2*)	(resid	23	and	name	HE2)	3.4	1.6	1.6

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assign  (resid  25  and  name  HD2* ) (resid  90  and  name  HA  )  3.9  2.1  2.1
assign  (resid  25  and  name  HD2* ) (resid  90  and  name  HB  )  3.9  2.1  2.1
assign  (resid  25  and  name  HD2* ) (resid  90  and  name  HG1* ) 2.9  1.1  1.1
assign  (resid  25  and  name  HN  ) (resid  23  and  name  HD2 )  3.9  2.1  2.1
assign  (resid  25  and  name  HN  ) (resid  23  and  name  HE2 )  3.9  2.1  2.1
assign  (resid  25  and  name  HN  ) (resid  24  and  name  HA  )  2.4  0.6  0.6
assign  (resid  25  and  name  HN  ) (resid  24  and  name  HG2* ) 3.4  1.6  1.6
assign  (resid  25  and  name  HN  ) (resid  24  and  name  HB  )  3.4  1.6  1.6
!GLY26
assign  (resid  26  and  name  HN  ) (resid  25  and  name  HA  )  3.9  2.1  2.1
assign  (resid  26  and  name  HN  ) (resid  25  and  name  HB1 )  2.9  1.1  1.1
assign  (resid  26  and  name  HN  ) (resid  25  and  name  HB2 )  3.4  1.6  1.6
assign  (resid  26  and  name  HN  ) (resid  27  and  name  HN  )  3.4  1.6  1.6
assign  (resid  26  and  name  HN  ) (resid  89  and  name  HN  )  3.4  1.6  1.6
assign  (resid  26  and  name  HN  ) (resid  89  and  name  HG1* ) 2.9  1.1  1.1
assign  (resid  26  and  name  HN  ) (resid  87  and  name  HG1* ) 3.4  1.6  1.6
assign  (resid  26  and  name  HN  ) (resid  87  and  name  HG2* ) 2.9  1.1  1.1
assign  (resid  26  and  name  HN  ) (resid  90  and  name  HA  )  3.4  1.6  1.6
!TYR27
assign  (resid  27  and  name  HD2 ) (resid  28  and  name  HB* )  3.9  2.1  2.1
assign  (resid  27  and  name  HD2 ) (resid  28  and  name  HG* )  3.9  2.1  2.1
assign  (resid  27  and  name  HD1 ) (resid  89  and  name  HG1* ) 2.9  1.1  1.1
assign  (resid  27  and  name  HD1 ) (resid  89  and  name  HG2* ) 2.9  1.1  1.1
assign  (resid  27  and  name  HD1 ) (resid  89  and  name  HB  )  3.9  2.1  2.1
assign  (resid  27  and  name  HE2 ) (resid  28  and  name  HA  )  3.9  2.1  2.1
assign  (resid  27  and  name  HE2 ) (resid  31  and  name  HG2* ) 2.9  1.1  1.1
assign  (resid  27  and  name  HE2 ) (resid  31  and  name  HG1* ) 3.9  2.1  2.1
assign  (resid  27  and  name  HE1 ) (resid  56  and  name  HD2* ) 2.9  1.1  1.1
assign  (resid  27  and  name  HE1 ) (resid  56  and  name  HD1* ) 3.9  2.1  2.1
assign  (resid  27  and  name  HN  ) (resid  26  and  name  HA* )  2.9  1.1  1.1
assign  (resid  27  and  name  HN  ) (resid  28  and  name  HA  )  3.9  2.1  2.1
assign  (resid  27  and  name  HN  ) (resid  28  and  name  HN  )  3.4  1.6  1.6
!LYS28
assign  (resid  28  and  name  HG* ) (resid  27  and  name  HB* )  3.9  2.1  2.1
assign  (resid  28  and  name  HN  ) (resid  27  and  name  HA  )  3.4  1.6  1.6
assign  (resid  28  and  name  HN  ) (resid  27  and  name  HB* )  2.9  1.1  1.1
assign  (resid  28  and  name  HN  ) (resid  27  and  name  HD2 )  2.9  1.1  1.1
assign  (resid  28  and  name  HN  ) (resid  27  and  name  HE2 )  3.9  2.1  2.1
assign  (resid  28  and  name  HN  ) (resid  29  and  name  HN  )  2.9  1.1  1.1
assign  (resid  28  and  name  HN  ) (resid  30  and  name  HN  )  3.9  2.1  2.1
assign  (resid  28  and  name  HN  ) (resid  31  and  name  HN  )  2.9  1.1  1.1
!SER29
assign  (resid  29  and  name  HA  ) (resid  32  and  name  HD* )  3.9  2.1  2.1
assign  (resid  29  and  name  HA  ) (resid  32  and  name  HB* )  3.4  1.6  1.6
assign  (resid  29  and  name  HN  ) (resid  28  and  name  HA  )  2.9  1.1  1.1
assign  (resid  29  and  name  HN  ) (resid  28  and  name  HB* )  2.9  1.1  1.1
assign  (resid  29  and  name  HN  ) (resid  28  and  name  HG* )  3.9  2.1  2.1
assign  (resid  29  and  name  HN  ) (resid  30  and  name  HN  )  2.4  0.6  0.6
!THR30
assign  (resid  30  and  name  HA  ) (resid  34  and  name  HD1* ) 3.4  1.6  1.6
assign  (resid  30  and  name  HA  ) (resid  33  and  name  HB2 )  3.4  1.6  1.6
assign  (resid  30  and  name  HB  ) (resid  29  and  name  HB* )  3.9  2.1  2.1
assign  (resid  30  and  name  HG2* ) (resid  27  and  name  HD2 )  3.9  2.1  2.1
assign  (resid  30  and  name  HN  ) (resid  28  and  name  HB* )  3.9  2.1  2.1
assign  (resid  30  and  name  HN  ) (resid  28  and  name  HG* )  3.9  2.1  2.1
assign  (resid  30  and  name  HN  ) (resid  29  and  name  HA  )  3.4  1.6  1.6
assign  (resid  30  and  name  HN  ) (resid  29  and  name  HB1 )  2.9  1.1  1.1
assign  (resid  30  and  name  HN  ) (resid  29  and  name  HB2 )  2.9  1.1  1.1
assign  (resid  30  and  name  HN  ) (resid  31  and  name  HN  )  3.4  1.6  1.6
!VAL31
assign  (resid  31  and  name  HA  ) (resid  34  and  name  HB* )  3.4  1.6  1.6
assign  (resid  31  and  name  HA  ) (resid  34  and  name  HD1* ) 2.9  1.1  1.1
assign  (resid  31  and  name  HA  ) (resid  34  and  name  HD2* ) 3.9  2.1  2.1
assign  (resid  31  and  name  HB  ) (resid  30  and  name  HN  )  3.9  2.1  2.1
assign  (resid  31  and  name  HB  ) (resid  32  and  name  HN  )  2.9  1.1  1.1
assign  (resid  31  and  name  HG1* ) (resid  32  and  name  HN  )  3.4  1.6  1.6
assign  (resid  31  and  name  HG1* ) (resid  35  and  name  HN  )  3.9  2.1  2.1
assign  (resid  31  and  name  HG1* ) (resid  35  and  name  HD* )  2.9  1.1  1.1
assign  (resid  31  and  name  HG1* ) (resid  35  and  name  HG* )  3.4  1.6  1.6
assign  (resid  31  and  name  HG1* ) (resid  56  and  name  HA  )  3.9  2.1  2.1
assign  (resid  31  and  name  HG1* ) (resid  34  and  name  HD1* ) 3.4  1.6  1.6
assign  (resid  31  and  name  HG1* ) (resid  59  and  name  HD1 )  3.4  1.6  1.6
assign  (resid  31  and  name  HG1* ) (resid  59  and  name  HE1 )  2.9  1.1  1.1
assign  (resid  31  and  name  HG2* ) (resid  59  and  name  HD1 )  2.9  1.1  1.1
assign  (resid  31  and  name  HG2* ) (resid  59  and  name  HE1 )  2.9  1.1  1.1
assign  (resid  31  and  name  HN  ) (resid  27  and  name  HA  )  3.4  1.6  1.6
assign  (resid  31  and  name  HN  ) (resid  30  and  name  HA  )  2.9  1.1  1.1
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assign	(resid	31	and	name	HN	)	(resid	30	and	name	HB	)	3.4	1.6	1.6
assign	(resid	31	and	name	HN	)	(resid	30	and	name	HG2*	)	2.9	1.1	1.1
assign	(resid	31	and	name	HN	)	(resid	32	and	name	HN	)	2.9	1.1	1.1
assign	(resid	31	and	name	HN	)	(resid	34	and	name	HN	)	3.9	2.1	2.1
assign	(resid	31	and	name	HN	)	(resid	56	and	name	HD2*	)	3.9	2.1	2.1
assign	(resid	31	and	name	HN	)	(resid	89	and	name	HG1*	)	3.9	2.1	2.1

!LYS32

assign	(resid	32	and	name	HA	)	(resid	36	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	32	and	name	HA	)	(resid	35	and	name	HB*	)	2.9	1.1	1.1
assign	(resid	32	and	name	HA	)	(resid	35	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	32	and	name	HG*	)	(resid	29	and	name	HA	)	3.9	2.1	2.1
assign	(resid	32	and	name	HG*	)	(resid	29	and	name	HB*	)	3.9	2.1	2.1
assign	(resid	32	and	name	HE*	)	(resid	36	and	name	HN	)	3.9	2.1	2.1
assign	(resid	32	and	name	HN	)	(resid	29	and	name	HA	)	2.9	1.1	1.1
assign	(resid	32	and	name	HN	)	(resid	31	and	name	HG1*	)	2.9	1.1	1.1
assign	(resid	32	and	name	HN	)	(resid	31	and	name	HG2*	)	3.4	1.6	1.6
assign	(resid	32	and	name	HN	)	(resid	31	and	name	HA	)	3.4	1.6	1.6
assign	(resid	32	and	name	HN	)	(resid	33	and	name	HN	)	2.9	1.1	1.1
assign	(resid	32	and	name	HN	)	(resid	34	and	name	HN	)	3.4	1.6	1.6

!SER33

assign	(resid	33	and	name	HA	)	(resid	36	and	name	HB2	)	2.9	1.1	1.1
assign	(resid	33	and	name	HA	)	(resid	36	and	name	HB1	)	3.4	1.6	1.6
assign	(resid	33	and	name	HA	)	(resid	36	and	name	HG*	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	30	and	name	HA	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	32	and	name	HA	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	32	and	name	HB*	)	2.9	1.1	1.1
assign	(resid	33	and	name	HN	)	(resid	32	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	33	and	name	HN	)	(resid	34	and	name	HN	)	2.4	0.6	0.6

!LEU34

assign	(resid	34	and	name	HA	)	(resid	37	and	name	HA*	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD1*	)	(resid	31	and	name	HA	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD1*	)	(resid	35	and	name	HN	)	3.4	1.6	1.6
assign	(resid	34	and	name	HD1*	)	(resid	59	and	name	HB1	)	3.4	1.6	1.6
assign	(resid	34	and	name	HD1*	)	(resid	59	and	name	HE1	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD2*	)	(resid	24	and	name	HB	)	2.9	1.1	1.1
assign	(resid	34	and	name	HD2*	)	(resid	24	and	name	HG2*	)	3.4	1.6	1.6
assign	(resid	34	and	name	HD2*	)	(resid	59	and	name	HD1	)	3.9	2.1	2.1
assign	(resid	34	and	name	HD2*	)	(resid	59	and	name	HE1	)	3.9	2.1	2.1
assign	(resid	34	and	name	HD2*	)	(resid	37	and	name	HA*	)	3.9	2.1	2.1
assign	(resid	34	and	name	HN	)	(resid	30	and	name	HA	)	2.9	1.1	1.1
assign	(resid	34	and	name	HN	)	(resid	30	and	name	HB	)	3.9	2.1	2.1
assign	(resid	34	and	name	HN	)	(resid	30	and	name	HG2*	)	3.4	1.6	1.6
assign	(resid	34	and	name	HN	)	(resid	31	and	name	HA	)	2.9	1.1	1.1
assign	(resid	34	and	name	HN	)	(resid	31	and	name	HB	)	3.9	2.1	2.1
assign	(resid	34	and	name	HN	)	(resid	32	and	name	HA	)	3.4	1.6	1.6
assign	(resid	34	and	name	HN	)	(resid	33	and	name	HB1	)	3.4	1.6	1.6
assign	(resid	34	and	name	HN	)	(resid	35	and	name	HN	)	2.9	1.1	1.1

!ARG35

assign	(resid	35	and	name	HA	)	(resid	36	and	name	HN	)	3.4	1.6	1.6
assign	(resid	35	and	name	HA	)	(resid	38	and	name	HB*	)	3.9	2.1	2.1
assign	(resid	35	and	name	HA	)	(resid	39	and	name	HB*	)	3.9	2.1	2.1
assign	(resid	35	and	name	HE	)	(resid	31	and	name	HG1*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HE	)	(resid	32	and	name	HA	)	3.9	2.1	2.1
assign	(resid	35	and	name	HE	)	(resid	58	and	name	HE2	)	3.9	2.1	2.1
assign	(resid	35	and	name	HE	)	(resid	55	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HD*	)	(resid	31	and	name	HG1*	)	3.4	1.6	1.6
assign	(resid	35	and	name	HD*	)	(resid	58	and	name	HD2	)	3.4	1.6	1.6
assign	(resid	35	and	name	HD*	)	(resid	58	and	name	HE2	)	2.9	1.1	1.1
assign	(resid	35	and	name	HD*	)	(resid	59	and	name	HE2	)	3.9	2.1	2.1
assign	(resid	35	and	name	HN	)	(resid	32	and	name	HA	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	34	and	name	HA	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	34	and	name	HB*	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	34	and	name	HG	)	3.4	1.6	1.6
assign	(resid	35	and	name	HN	)	(resid	36	and	name	HN	)	2.9	1.1	1.1
assign	(resid	35	and	name	HN	)	(resid	59	and	name	HD1	)	3.9	2.1	2.1
assign	(resid	35	and	name	HN	)	(resid	59	and	name	HE1	)	3.9	2.1	2.1

!GLN36

assign	(resid	36	and	name	HA	)	(resid	37	and	name	HA*	)	3.9	2.1	2.1
assign	(resid	36	and	name	HB1	)	(resid	37	and	name	HN	)	3.4	1.6	1.6
assign	(resid	36	and	name	HB2	)	(resid	37	and	name	HN	)	3.4	1.6	1.6
assign	(resid	36	and	name	HE2*	)	(resid	32	and	name	HB*	)	3.9	2.1	2.1
assign	(resid	36	and	name	HE2*	)	(resid	32	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	36	and	name	HE2*	)	(resid	33	and	name	HA	)	3.9	2.1	2.1
assign	(resid	36	and	name	HE2*	)	(resid	35	and	name	HA	)	3.9	2.1	2.1
assign	(resid	36	and	name	HE2*	)	(resid	37	and	name	HN	)	3.9	2.1	2.1
assign	(resid	36	and	name	HN	)	(resid	32	and	name	HA	)	3.4	1.6	1.6
assign	(resid	36	and	name	HN	)	(resid	33	and	name	HA	)	3.4	1.6	1.6

assign	(resid	36	and	name	HN)	(resid	35	and	name	HB*)	3.4	1.6	1.6
assign	(resid	36	and	name	HN)	(resid	37	and	name	HA*)	3.9	2.1	2.1
assign	(resid	36	and	name	HN)	(resid	37	and	name	HN)	2.9	1.1	1.1
assign	(resid	36	and	name	HN)	(resid	38	and	name	HN)	3.4	1.6	1.6

!GLY37

assign	(resid	37	and	name	HA*)	(resid	38	and	name	HG*)	3.9	2.1	2.1
assign	(resid	37	and	name	HA*)	(resid	38	and	name	HN)	3.9	2.1	2.1
assign	(resid	37	and	name	HN)	(resid	33	and	name	HA)	3.4	1.6	1.6
assign	(resid	37	and	name	HN)	(resid	34	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	37	and	name	HN)	(resid	35	and	name	HN)	3.9	2.1	2.1
assign	(resid	37	and	name	HN)	(resid	36	and	name	HA)	2.9	1.1	1.1
assign	(resid	37	and	name	HN)	(resid	36	and	name	HG*)	3.9	2.1	2.1
assign	(resid	37	and	name	HN)	(resid	38	and	name	HN)	2.9	1.1	1.1
assign	(resid	37	and	name	HN)	(resid	39	and	name	HN)	3.9	2.1	2.1

!LYS38

assign	(resid	38	and	name	HA)	(resid	37	and	name	HN)	3.9	2.1	2.1
assign	(resid	38	and	name	HB*)	(resid	62	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	38	and	name	HG*)	(resid	35	and	name	HA)	3.9	2.1	2.1
assign	(resid	38	and	name	HN)	(resid	35	and	name	HA)	3.9	2.1	2.1
assign	(resid	38	and	name	HN)	(resid	39	and	name	HN)	2.9	1.1	1.1
assign	(resid	38	and	name	HN)	(resid	62	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	38	and	name	HN)	(resid	62	and	name	HB*)	3.9	2.1	2.1

!SER39

assign	(resid	39	and	name	HA)	(resid	40	and	name	HN)	2.9	1.1	1.1
assign	(resid	39	and	name	HA)	(resid	41	and	name	HN)	3.9	2.1	2.1
assign	(resid	39	and	name	HA)	(resid	93	and	name	HA)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	35	and	name	HA)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	63	and	name	HB*)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	38	and	name	HA)	2.9	1.1	1.1
assign	(resid	39	and	name	HN)	(resid	38	and	name	HB*)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	38	and	name	HG*)	3.9	2.1	2.1
assign	(resid	39	and	name	HN)	(resid	40	and	name	HN)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	62	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	39	and	name	HN)	(resid	93	and	name	HD2*)	3.9	2.1	2.1

!LYS40

assign	(resid	40	and	name	HA)	(resid	39	and	name	HA)	3.9	2.1	2.1
assign	(resid	40	and	name	HB*)	(resid	39	and	name	HA)	3.9	2.1	2.1
assign	(resid	40	and	name	HB*)	(resid	41	and	name	HB*)	3.9	2.1	2.1
assign	(resid	40	and	name	HE*)	(resid	64	and	name	HN)	3.4	1.6	1.6
assign	(resid	40	and	name	HE*)	(resid	93	and	name	HA)	3.4	1.6	1.6
assign	(resid	40	and	name	HN)	(resid	41	and	name	HN)	2.4	0.6	0.6
assign	(resid	40	and	name	HN)	(resid	93	and	name	HD*)	2.9	1.1	1.1

!LEU41

assign	(resid	41	and	name	HD2*)	(resid	43	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	41	and	name	HD2*)	(resid	67	and	name	HA)	3.4	1.6	1.6
assign	(resid	41	and	name	HD2*)	(resid	68	and	name	HN)	3.4	1.6	1.6
assign	(resid	41	and	name	HD2*)	(resid	68	and	name	HB1)	2.9	1.1	1.1
assign	(resid	41	and	name	HD2*)	(resid	68	and	name	HB2)	2.9	1.1	1.1
assign	(resid	41	and	name	HD2*)	(resid	68	and	name	HD1)	3.4	1.6	1.6
assign	(resid	41	and	name	HD2*)	(resid	42	and	name	HN)	2.4	0.6	0.6
assign	(resid	41	and	name	HD2*)	(resid	66	and	name	HN)	3.4	1.6	1.6
assign	(resid	41	and	name	HD1*)	(resid	68	and	name	HB*)	3.9	2.1	2.1
assign	(resid	41	and	name	HD1*)	(resid	68	and	name	HD1)	3.9	2.1	2.1
assign	(resid	41	and	name	HD1*)	(resid	42	and	name	HN)	3.4	1.6	1.6
assign	(resid	41	and	name	HN)	(resid	40	and	name	HA)	2.9	1.1	1.1
assign	(resid	41	and	name	HN)	(resid	40	and	name	HB*)	2.9	1.1	1.1
assign	(resid	41	and	name	HN)	(resid	40	and	name	HG*)	3.4	1.6	1.6
assign	(resid	41	and	name	HN)	(resid	92	and	name	HN)	3.4	1.6	1.6
assign	(resid	41	and	name	HN)	(resid	42	and	name	HN)	3.9	2.1	2.1

!ILE42

assign	(resid	42	and	name	HA)	(resid	43	and	name	HN)	2.4	0.6	0.6
assign	(resid	42	and	name	HA)	(resid	91	and	name	HA)	2.4	0.6	0.6
assign	(resid	42	and	name	HB)	(resid	43	and	name	HN)	2.9	1.1	1.1
assign	(resid	42	and	name	HD1*)	(resid	43	and	name	HN)	3.9	2.1	2.1
assign	(resid	42	and	name	HD1*)	(resid	60	and	name	HA)	3.4	1.6	1.6
assign	(resid	42	and	name	HD1*)	(resid	67	and	name	HA)	2.9	1.1	1.1
assign	(resid	42	and	name	HD1*)	(resid	67	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	42	and	name	HG2*)	(resid	91	and	name	HB2)	3.9	2.1	2.1
assign	(resid	42	and	name	HG2*)	(resid	91	and	name	HA)	3.4	1.6	1.6
assign	(resid	42	and	name	HG2*)	(resid	92	and	name	HN)	2.9	1.1	1.1
assign	(resid	42	and	name	HN)	(resid	41	and	name	HA)	2.4	0.6	0.6
assign	(resid	42	and	name	HN)	(resid	41	and	name	HB*)	3.9	2.1	2.1
assign	(resid	42	and	name	HN)	(resid	67	and	name	HA)	2.9	1.1	1.1
assign	(resid	42	and	name	HN)	(resid	67	and	name	HG2*)	3.4	1.6	1.6

!ILE43

assign	(resid	43	and	name	HA)	(resid	42	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	43	and	name	HA)	(resid	68	and	name	HB2)	3.4	1.6	1.6

assign	(resid	43	and	name	HA)	(resid	68	and	name	HB1)	3.4	1.6	1.6
assign	(resid	43	and	name	HA)	(resid	68	and	name	HD2)	3.4	1.6	1.6
assign	(resid	43	and	name	HB)	(resid	90	and	name	HN)	3.4	1.6	1.6
assign	(resid	43	and	name	HD1*)	(resid	41	and	name	HG)	3.4	1.6	1.6
assign	(resid	43	and	name	HD1*)	(resid	41	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	43	and	name	HD1*)	(resid	41	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	43	and	name	HD1*)	(resid	44	and	name	HN)	3.9	2.1	2.1
assign	(resid	43	and	name	HD1*)	(resid	68	and	name	HB*)	3.9	2.1	2.1
assign	(resid	43	and	name	HD1*)	(resid	68	and	name	HD2)	2.9	1.1	1.1
assign	(resid	43	and	name	HD1*)	(resid	68	and	name	HN)	3.4	1.6	1.6
assign	(resid	43	and	name	HD1*)	(resid	70	and	name	HD1)	3.9	2.1	2.1
assign	(resid	43	and	name	HD1*)	(resid	70	and	name	HE1)	3.9	2.1	2.1
assign	(resid	43	and	name	HG1*)	(resid	68	and	name	HD2)	3.9	2.1	2.1
assign	(resid	43	and	name	HG2*)	(resid	44	and	name	HN)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	68	and	name	HB*)	3.4	1.6	1.6
assign	(resid	43	and	name	HG2*)	(resid	68	and	name	HD2)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	68	and	name	HE2)	3.4	1.6	1.6
assign	(resid	43	and	name	HG2*)	(resid	69	and	name	HA)	3.9	2.1	2.1
assign	(resid	43	and	name	HG2*)	(resid	70	and	name	HB*)	3.9	2.1	2.1
assign	(resid	43	and	name	HG2*)	(resid	70	and	name	HD1)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	70	and	name	HE1)	2.9	1.1	1.1
assign	(resid	43	and	name	HG2*)	(resid	77	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	43	and	name	HN)	(resid	42	and	name	HN)	3.9	2.1	2.1
assign	(resid	43	and	name	HN)	(resid	91	and	name	HA)	2.9	1.1	1.1

!ILE44

assign	(resid	44	and	name	HA)	(resid	43	and	name	HB)	3.9	2.1	2.1
assign	(resid	44	and	name	HA)	(resid	89	and	name	HA)	2.4	0.6	0.6
assign	(resid	44	and	name	HD1*)	(resid	45	and	name	HN)	2.9	1.1	1.1
assign	(resid	44	and	name	HD1*)	(resid	53	and	name	HA)	2.9	1.1	1.1
assign	(resid	44	and	name	HD1*)	(resid	67	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	44	and	name	HD1*)	(resid	69	and	name	HA)	3.4	1.6	1.6
assign	(resid	44	and	name	HD1*)	(resid	69	and	name	HD2)	2.9	1.1	1.1
assign	(resid	44	and	name	HD1*)	(resid	69	and	name	HE2)	2.9	1.1	1.1
assign	(resid	44	and	name	HG2*)	(resid	45	and	name	HN)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	53	and	name	HA)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	56	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	56	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	69	and	name	HD2)	3.4	1.6	1.6
assign	(resid	44	and	name	HG2*)	(resid	69	and	name	HE2)	2.9	1.1	1.1
assign	(resid	44	and	name	HG2*)	(resid	89	and	name	HA)	2.9	1.1	1.1
assign	(resid	44	and	name	HG2*)	(resid	89	and	name	HB)	3.9	2.1	2.1
assign	(resid	44	and	name	HN)	(resid	43	and	name	HA)	2.4	0.6	0.6
assign	(resid	44	and	name	HN)	(resid	43	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	44	and	name	HN)	(resid	43	and	name	HN)	3.9	2.1	2.1
assign	(resid	44	and	name	HN)	(resid	69	and	name	HA)	2.4	0.6	0.6
assign	(resid	44	and	name	HN)	(resid	68	and	name	HB*)	3.4	1.6	1.6
assign	(resid	44	and	name	HN)	(resid	69	and	name	HE2)	2.9	1.1	1.1
assign	(resid	44	and	name	HN)	(resid	69	and	name	HD2)	3.4	1.6	1.6

!ALA45

assign	(resid	45	and	name	HA)	(resid	44	and	name	HB)	3.9	2.1	2.1
assign	(resid	45	and	name	HA)	(resid	44	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	45	and	name	HA)	(resid	46	and	name	HB*)	3.4	1.6	1.6
assign	(resid	45	and	name	HB*)	(resid	46	and	name	HB*)	3.9	2.1	2.1
assign	(resid	45	and	name	HB*)	(resid	73	and	name	HA*)	2.9	1.1	1.1
assign	(resid	45	and	name	HB*)	(resid	70	and	name	HD2)	3.9	2.1	2.1
assign	(resid	45	and	name	HB*)	(resid	73	and	name	HN)	3.4	1.6	1.6
assign	(resid	45	and	name	HB*)	(resid	74	and	name	HN)	3.4	1.6	1.6
assign	(resid	45	and	name	HB*)	(resid	77	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	45	and	name	HB*)	(resid	77	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	45	and	name	HB*)	(resid	77	and	name	HG)	3.9	2.1	2.1
assign	(resid	45	and	name	HB*)	(resid	88	and	name	HA*)	3.4	1.6	1.6
assign	(resid	45	and	name	HN)	(resid	44	and	name	HA)	2.4	0.6	0.6
assign	(resid	45	and	name	HN)	(resid	44	and	name	HB)	2.9	1.1	1.1
assign	(resid	45	and	name	HN)	(resid	44	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	45	and	name	HN)	(resid	44	and	name	HN)	3.4	1.6	1.6
assign	(resid	45	and	name	HN)	(resid	46	and	name	HN)	3.4	1.6	1.6
assign	(resid	45	and	name	HN)	(resid	77	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	45	and	name	HN)	(resid	88	and	name	HA*)	3.9	2.1	2.1

!ALA46

assign	(resid	46	and	name	HA)	(resid	47	and	name	HN)	2.9	1.1	1.1
assign	(resid	46	and	name	HA)	(resid	48	and	name	HN)	2.9	1.1	1.1
assign	(resid	46	and	name	HB*)	(resid	47	and	name	HN)	2.9	1.1	1.1
assign	(resid	46	and	name	HB*)	(resid	48	and	name	HN)	3.4	1.6	1.6
assign	(resid	46	and	name	HN)	(resid	44	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	46	and	name	HN)	(resid	45	and	name	HA)	3.9	2.1	2.1
assign	(resid	46	and	name	HN)	(resid	45	and	name	HB*)	2.9	1.1	1.1
assign	(resid	46	and	name	HN)	(resid	47	and	name	HN)	3.4	1.6	1.6

assign	(resid	46	and	name	HN	)	(resid	69	and	name	HB1)	3.4	1.6	1.6
assign	(resid	46	and	name	HN	)	(resid	70	and	name	HD2)	2.9	1.1	1.1
assign	(resid	46	and	name	HN	)	(resid	70	and	name	HN)	3.4	1.6	1.6
assign	(resid	46	and	name	HN	)	(resid	71	and	name	HA)	3.9	2.1	2.1
assign	(resid	46	and	name	HN	)	(resid	73	and	name	HA*)	2.9	1.1	1.1
assign	(resid	46	and	name	HN	)	(resid	74	and	name	HN)	3.9	2.1	2.1

!ASN47

assign	(resid	47	and	name	HN	)	(resid	48	and	name	HB)	3.9	2.1	2.1
assign	(resid	47	and	name	HN	)	(resid	71	and	name	HA)	3.9	2.1	2.1
assign	(resid	47	and	name	HN	)	(resid	73	and	name	HA*)	3.9	2.1	2.1
assign	(resid	47	and	name	HN	)	(resid	48	and	name	HN)	2.9	1.1	1.1

!THR48

assign	(resid	48	and	name	HA	)	(resid	49	and	name	HB*)	3.9	2.1	2.1
assign	(resid	48	and	name	HA	)	(resid	49	and	name	HD*)	2.4	0.6	0.6
assign	(resid	48	and	name	HA	)	(resid	49	and	name	HG*)	3.9	2.1	2.1
assign	(resid	48	and	name	HB	)	(resid	44	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	48	and	name	HB	)	(resid	47	and	name	HB2)	3.4	1.6	1.6
assign	(resid	48	and	name	HB	)	(resid	47	and	name	HB1)	3.4	1.6	1.6
assign	(resid	48	and	name	HG2*	)	(resid	44	and	name	HB)	2.9	1.1	1.1
assign	(resid	48	and	name	HG2*	)	(resid	44	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	48	and	name	HG2*	)	(resid	49	and	name	HD2)	2.9	1.1	1.1
assign	(resid	48	and	name	HG2*	)	(resid	52	and	name	HN)	3.4	1.6	1.6
assign	(resid	48	and	name	HG2*	)	(resid	52	and	name	HG*)	3.4	1.6	1.6
assign	(resid	48	and	name	HG2*	)	(resid	53	and	name	HN)	2.9	1.1	1.1
assign	(resid	48	and	name	HN	)	(resid	47	and	name	HA)	3.4	1.6	1.6
assign	(resid	48	and	name	HN	)	(resid	47	and	name	HB2)	2.9	1.1	1.1
assign	(resid	48	and	name	HN	)	(resid	47	and	name	HB1)	3.4	1.6	1.6
assign	(resid	48	and	name	HN	)	(resid	49	and	name	HD*)	3.9	2.1	2.1

!PRO49

assign	(resid	49	and	name	HA	)	(resid	48	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	49	and	name	HA	)	(resid	50	and	name	HB)	3.9	2.1	2.1
assign	(resid	49	and	name	HA	)	(resid	50	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	49	and	name	HA	)	(resid	50	and	name	HN)	2.4	0.6	0.6

!VAL50

assign	(resid	50	and	name	HA	)	(resid	51	and	name	HB*)	3.9	2.1	2.1
assign	(resid	50	and	name	HA	)	(resid	53	and	name	HB*)	3.4	1.6	1.6
assign	(resid	50	and	name	HA	)	(resid	53	and	name	HG*)	3.9	2.1	2.1
assign	(resid	50	and	name	HB	)	(resid	49	and	name	HG*)	3.9	2.1	2.1
assign	(resid	50	and	name	HB	)	(resid	51	and	name	HN)	2.9	1.1	1.1
assign	(resid	50	and	name	HG1*	)	(resid	51	and	name	HN)	2.9	1.1	1.1
assign	(resid	50	and	name	HG2*	)	(resid	51	and	name	HN)	2.9	1.1	1.1
assign	(resid	50	and	name	HN	)	(resid	49	and	name	HB*)	3.4	1.6	1.6
assign	(resid	50	and	name	HN	)	(resid	51	and	name	HN)	3.4	1.6	1.6

!LEU51

assign	(resid	51	and	name	HA	)	(resid	54	and	name	HB*)	3.9	2.1	2.1
assign	(resid	51	and	name	HB*	)	(resid	52	and	name	HN)	2.9	1.1	1.1
assign	(resid	51	and	name	HB*	)	(resid	54	and	name	HB*)	3.9	2.1	2.1
assign	(resid	51	and	name	HD1*	)	(resid	50	and	name	HN)	3.9	2.1	2.1
assign	(resid	51	and	name	HD1*	)	(resid	52	and	name	HN)	3.4	1.6	1.6
assign	(resid	51	and	name	HD2*	)	(resid	50	and	name	HN)	3.9	2.1	2.1
assign	(resid	51	and	name	HD2*	)	(resid	52	and	name	HN)	3.4	1.6	1.6
assign	(resid	51	and	name	HN	)	(resid	48	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	51	and	name	HN	)	(resid	50	and	name	HA)	3.4	1.6	1.6
assign	(resid	51	and	name	HN	)	(resid	52	and	name	HN)	2.9	1.1	1.1
assign	(resid	51	and	name	HN	)	(resid	53	and	name	HN)	3.9	2.1	2.1

!ARG52

assign	(resid	52	and	name	HA	)	(resid	56	and	name	HB2)	2.9	1.1	1.1
assign	(resid	52	and	name	HA	)	(resid	56	and	name	HG)	3.9	2.1	2.1
assign	(resid	52	and	name	HA	)	(resid	55	and	name	HB*)	3.4	1.6	1.6
assign	(resid	52	and	name	HD*	)	(resid	51	and	name	HA)	3.9	2.1	2.1
assign	(resid	52	and	name	HN	)	(resid	50	and	name	HA)	3.9	2.1	2.1
assign	(resid	52	and	name	HN	)	(resid	50	and	name	HN)	2.9	1.1	1.1
assign	(resid	52	and	name	HN	)	(resid	51	and	name	HA)	2.9	1.1	1.1
assign	(resid	52	and	name	HN	)	(resid	53	and	name	HN)	2.4	0.6	0.6

!LYS53

assign	(resid	53	and	name	HA	)	(resid	54	and	name	HN)	2.9	1.1	1.1
assign	(resid	53	and	name	HA	)	(resid	56	and	name	HB*)	3.4	1.6	1.6
assign	(resid	53	and	name	HD*	)	(resid	50	and	name	HA)	3.9	2.1	2.1
assign	(resid	53	and	name	HD*	)	(resid	54	and	name	HN)	3.9	2.1	2.1
assign	(resid	53	and	name	HD*	)	(resid	57	and	name	HG*)	3.4	1.6	1.6
assign	(resid	53	and	name	HD*	)	(resid	69	and	name	HE1)	3.4	1.6	1.6
assign	(resid	53	and	name	HE*	)	(resid	69	and	name	HE1)	3.4	1.6	1.6
assign	(resid	53	and	name	HE*	)	(resid	69	and	name	HD1)	3.9	2.1	2.1
assign	(resid	53	and	name	HG*	)	(resid	54	and	name	HN)	3.4	1.6	1.6
assign	(resid	53	and	name	HG*	)	(resid	56	and	name	HG*)	3.9	2.1	2.1
assign	(resid	53	and	name	HG*	)	(resid	69	and	name	HE1)	3.9	2.1	2.1
assign	(resid	53	and	name	HN	)	(resid	50	and	name	HA)	2.4	0.6	0.6

assign	(resid	53	and	name	HN)	(resid	50	and	name	HB)	3.9	2.1	2.1
assign	(resid	53	and	name	HN)	(resid	52	and	name	HA)	2.9	1.1	1.1
assign	(resid	53	and	name	HN)	(resid	54	and	name	HN)	2.9	1.1	1.1
assign	(resid	53	and	name	HN)	(resid	55	and	name	HN)	3.9	2.1	2.1
assign	(resid	53	and	name	HN)	(resid	44	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	53	and	name	HN)	(resid	44	and	name	HD1*)	3.4	1.6	1.6

!SER54

assign	(resid	54	and	name	HA)	(resid	57	and	name	HB1)	2.9	1.1	1.1
assign	(resid	54	and	name	HA)	(resid	57	and	name	HB2)	2.9	1.1	1.1
assign	(resid	54	and	name	HA)	(resid	57	and	name	HG*)	3.9	2.1	2.1
assign	(resid	54	and	name	HB*)	(resid	55	and	name	HN)	2.9	1.1	1.1
assign	(resid	54	and	name	HN)	(resid	50	and	name	HA)	2.9	1.1	1.1
assign	(resid	54	and	name	HN)	(resid	50	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	54	and	name	HN)	(resid	51	and	name	HA)	2.9	1.1	1.1
assign	(resid	54	and	name	HN)	(resid	51	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	54	and	name	HN)	(resid	51	and	name	HN)	3.9	2.1	2.1
assign	(resid	54	and	name	HN)	(resid	53	and	name	HB*)	2.9	1.1	1.1
assign	(resid	54	and	name	HN)	(resid	57	and	name	HG*)	3.9	2.1	2.1
assign	(resid	54	and	name	HN)	(resid	55	and	name	HN)	2.9	1.1	1.1

!GLU55

assign	(resid	55	and	name	HA)	(resid	56	and	name	HN)	2.9	1.1	1.1
assign	(resid	55	and	name	HA)	(resid	58	and	name	HB1)	2.9	1.1	1.1
assign	(resid	55	and	name	HA)	(resid	58	and	name	HB2)	2.9	1.1	1.1
assign	(resid	55	and	name	HB1)	(resid	56	and	name	HN)	2.9	1.1	1.1
assign	(resid	55	and	name	HB2)	(resid	56	and	name	HN)	3.4	1.6	1.6
assign	(resid	55	and	name	HB1)	(resid	59	and	name	HN)	3.9	2.1	2.1
assign	(resid	55	and	name	HG*)	(resid	59	and	name	HN)	3.9	2.1	2.1
assign	(resid	55	and	name	HG*)	(resid	59	and	name	HD2)	3.4	1.6	1.6
assign	(resid	55	and	name	HG*)	(resid	59	and	name	HE2)	2.9	1.1	1.1
assign	(resid	55	and	name	HN)	(resid	52	and	name	HA)	2.9	1.1	1.1
assign	(resid	55	and	name	HN)	(resid	54	and	name	HA)	3.4	1.6	1.6
assign	(resid	55	and	name	HN)	(resid	56	and	name	HN)	2.9	1.1	1.1

!LEU56

assign	(resid	56	and	name	HA)	(resid	59	and	name	HB2)	2.9	1.1	1.1
assign	(resid	56	and	name	HD1*)	(resid	44	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	56	and	name	HD1*)	(resid	53	and	name	HA)	3.4	1.6	1.6
assign	(resid	56	and	name	HD1*)	(resid	57	and	name	HN)	3.4	1.6	1.6
assign	(resid	56	and	name	HD2*)	(resid	57	and	name	HN)	3.9	2.1	2.1
assign	(resid	56	and	name	HD2*)	(resid	31	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	56	and	name	HD2*)	(resid	31	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	56	and	name	HN)	(resid	27	and	name	HE1)	3.4	1.6	1.6
assign	(resid	56	and	name	HN)	(resid	53	and	name	HA)	3.4	1.6	1.6
assign	(resid	56	and	name	HN)	(resid	54	and	name	HA)	3.9	2.1	2.1
assign	(resid	56	and	name	HN)	(resid	55	and	name	HG*)	2.9	1.1	1.1
assign	(resid	56	and	name	HN)	(resid	57	and	name	HN)	2.9	1.1	1.1

!GLU57

assign	(resid	57	and	name	HA)	(resid	58	and	name	HN)	2.9	1.1	1.1
assign	(resid	57	and	name	HA)	(resid	60	and	name	HB*)	2.9	1.1	1.1
assign	(resid	57	and	name	HB2)	(resid	56	and	name	HA)	3.9	2.1	2.1
assign	(resid	57	and	name	HG*)	(resid	53	and	name	HD*)	3.4	1.6	1.6
assign	(resid	57	and	name	HN)	(resid	53	and	name	HB*)	3.9	2.1	2.1
assign	(resid	57	and	name	HN)	(resid	54	and	name	HA)	3.4	1.6	1.6
assign	(resid	57	and	name	HN)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	57	and	name	HN)	(resid	56	and	name	HB*)	2.9	1.1	1.1
assign	(resid	57	and	name	HN)	(resid	56	and	name	HG)	3.4	1.6	1.6
assign	(resid	57	and	name	HN)	(resid	67	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	57	and	name	HN)	(resid	58	and	name	HN)	2.9	1.1	1.1

!TYR58

assign	(resid	58	and	name	HA)	(resid	57	and	name	HN)	3.9	2.1	2.1
assign	(resid	58	and	name	HA)	(resid	61	and	name	HB1)	2.9	1.1	1.1
assign	(resid	58	and	name	HA)	(resid	61	and	name	HB2)	3.4	1.6	1.6
assign	(resid	58	and	name	HA)	(resid	61	and	name	HE*)	3.9	2.1	2.1
assign	(resid	58	and	name	HA)	(resid	61	and	name	HG*)	3.9	2.1	2.1
assign	(resid	58	and	name	HB*)	(resid	59	and	name	HN)	3.9	2.1	2.1
assign	(resid	58	and	name	HD2)	(resid	55	and	name	HA)	3.4	1.6	1.6
assign	(resid	58	and	name	HD2)	(resid	59	and	name	HA)	3.9	2.1	2.1
assign	(resid	58	and	name	HD2)	(resid	59	and	name	HD2)	3.4	1.6	1.6
assign	(resid	58	and	name	HD2)	(resid	59	and	name	HE2)	3.4	1.6	1.6
assign	(resid	58	and	name	HE1)	(resid	61	and	name	HE*)	2.9	1.1	1.1
assign	(resid	58	and	name	HE1)	(resid	61	and	name	HG*)	3.4	1.6	1.6
assign	(resid	58	and	name	HN)	(resid	55	and	name	HA)	2.9	1.1	1.1
assign	(resid	58	and	name	HN)	(resid	55	and	name	HB*)	3.9	2.1	2.1
assign	(resid	58	and	name	HN)	(resid	57	and	name	HB1)	3.4	1.6	1.6
assign	(resid	58	and	name	HN)	(resid	57	and	name	HB2)	3.4	1.6	1.6
assign	(resid	58	and	name	HN)	(resid	57	and	name	HG*)	3.9	2.1	2.1
assign	(resid	58	and	name	HN)	(resid	59	and	name	HN)	2.9	1.1	1.1
assign	(resid	58	and	name	HN)	(resid	60	and	name	HN)	3.4	1.6	1.6

!TYR59

assign	(resid	59	and	name	HA)	(resid	62	and	name	HG)	2.9	1.1	1.1
assign	(resid	59	and	name	HA)	(resid	62	and	name	HN)	2.9	1.1	1.1
assign	(resid	59	and	name	HB1)	(resid	42	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	59	and	name	HD1)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	59	and	name	HD2)	(resid	58	and	name	HE2)	3.9	2.1	2.1
assign	(resid	59	and	name	HE1)	(resid	31	and	name	HB)	3.4	1.6	1.6
assign	(resid	59	and	name	HN)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	56	and	name	HB*)	3.9	2.1	2.1
assign	(resid	59	and	name	HN)	(resid	58	and	name	HA)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	58	and	name	HD2)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	60	and	name	HN)	2.9	1.1	1.1
assign	(resid	59	and	name	HN)	(resid	55	and	name	HG*)	3.9	2.1	2.1
assign	(resid	59	and	name	HN)	(resid	55	and	name	HB*)	3.9	2.1	2.1

!ALA60

assign	(resid	60	and	name	HA)	(resid	63	and	name	HB*)	3.9	2.1	2.1
assign	(resid	60	and	name	HA)	(resid	65	and	name	HB)	2.9	1.1	1.1
assign	(resid	60	and	name	HA)	(resid	65	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	60	and	name	HA)	(resid	65	and	name	HN)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	57	and	name	HB1)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	57	and	name	HB2)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	61	and	name	HN)	2.9	1.1	1.1
assign	(resid	60	and	name	HB*)	(resid	65	and	name	HB)	3.4	1.6	1.6
assign	(resid	60	and	name	HB*)	(resid	65	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	65	and	name	HN)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	67	and	name	HB)	3.4	1.6	1.6
assign	(resid	60	and	name	HB*)	(resid	67	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	60	and	name	HB*)	(resid	67	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	60	and	name	HN)	(resid	42	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	42	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	56	and	name	HA)	2.9	1.1	1.1
assign	(resid	60	and	name	HN)	(resid	57	and	name	HA)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	59	and	name	HA)	2.9	1.1	1.1
assign	(resid	60	and	name	HN)	(resid	59	and	name	HB1)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	59	and	name	HB2)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	59	and	name	HD1)	3.4	1.6	1.6
assign	(resid	60	and	name	HN)	(resid	61	and	name	HA)	3.9	2.1	2.1
assign	(resid	60	and	name	HN)	(resid	61	and	name	HN)	2.9	1.1	1.1

!MET61

assign	(resid	61	and	name	HA)	(resid	62	and	name	HA)	3.4	1.6	1.6
assign	(resid	61	and	name	HA)	(resid	62	and	name	HN)	2.9	1.1	1.1
assign	(resid	61	and	name	HB2)	(resid	60	and	name	HB*)	3.9	2.1	2.1
assign	(resid	61	and	name	HB1)	(resid	62	and	name	HN)	2.9	1.1	1.1
assign	(resid	61	and	name	HB2)	(resid	62	and	name	HN)	3.4	1.6	1.6
assign	(resid	61	and	name	HE*)	(resid	62	and	name	HA)	3.9	2.1	2.1
assign	(resid	61	and	name	HE*)	(resid	62	and	name	HN)	3.9	2.1	2.1
assign	(resid	61	and	name	HG*)	(resid	62	and	name	HN)	3.9	2.1	2.1
assign	(resid	61	and	name	HN)	(resid	57	and	name	HA)	3.9	2.1	2.1
assign	(resid	61	and	name	HN)	(resid	58	and	name	HA)	3.4	1.6	1.6
assign	(resid	61	and	name	HN)	(resid	58	and	name	HD1)	3.9	2.1	2.1
assign	(resid	61	and	name	HN)	(resid	60	and	name	HA)	2.9	1.1	1.1
assign	(resid	61	and	name	HN)	(resid	64	and	name	HA)	3.9	2.1	2.1
assign	(resid	61	and	name	HN)	(resid	62	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	61	and	name	HN)	(resid	62	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	61	and	name	HN)	(resid	62	and	name	HN)	2.9	1.1	1.1
assign	(resid	61	and	name	HN)	(resid	63	and	name	HN)	3.9	2.1	2.1

!LEU62

assign	(resid	62	and	name	HG)	(resid	59	and	name	HD2)	3.9	2.1	2.1
assign	(resid	62	and	name	HG)	(resid	61	and	name	HE*)	3.9	2.1	2.1
assign	(resid	62	and	name	HD1*)	(resid	63	and	name	HN)	3.4	1.6	1.6
assign	(resid	62	and	name	HD1*)	(resid	58	and	name	HD2)	3.4	1.6	1.6
assign	(resid	62	and	name	HD1*)	(resid	58	and	name	HE2)	2.9	1.1	1.1
assign	(resid	62	and	name	HD1*)	(resid	59	and	name	HA)	2.9	1.1	1.1
assign	(resid	62	and	name	HD1*)	(resid	59	and	name	HD2)	2.9	1.1	1.1
assign	(resid	62	and	name	HD1*)	(resid	59	and	name	HE2)	3.4	1.6	1.6
assign	(resid	62	and	name	HD2*)	(resid	58	and	name	HD2)	3.4	1.6	1.6
assign	(resid	62	and	name	HD2*)	(resid	58	and	name	HE2)	2.9	1.1	1.1
assign	(resid	62	and	name	HD2*)	(resid	59	and	name	HA)	2.9	1.1	1.1
assign	(resid	62	and	name	HD2*)	(resid	59	and	name	HD2)	3.9	2.1	2.1
assign	(resid	62	and	name	HD2*)	(resid	61	and	name	HN)	2.9	1.1	1.1
assign	(resid	62	and	name	HD2*)	(resid	61	and	name	HE*)	3.4	1.6	1.6
assign	(resid	62	and	name	HD2*)	(resid	63	and	name	HN)	3.4	1.6	1.6
assign	(resid	62	and	name	HN)	(resid	58	and	name	HD2)	2.9	1.1	1.1
assign	(resid	62	and	name	HN)	(resid	59	and	name	HD2)	3.9	2.1	2.1
assign	(resid	62	and	name	HN)	(resid	60	and	name	HN)	3.9	2.1	2.1
assign	(resid	62	and	name	HN)	(resid	63	and	name	HN)	2.9	1.1	1.1

!SER63

assign	(resid	63	and	name	HA)	(resid	64	and	name	HB*)	3.9	2.1	2.1
assign	(resid	63	and	name	HN)	(resid	38	and	name	HA)	3.9	2.1	2.1
assign	(resid	63	and	name	HN)	(resid	60	and	name	HA)	3.4	1.6	1.6
assign	(resid	63	and	name	HN)	(resid	62	and	name	HA)	2.9	1.1	1.1
assign	(resid	63	and	name	HN)	(resid	62	and	name	HB1)	3.4	1.6	1.6
assign	(resid	63	and	name	HN)	(resid	62	and	name	HB2)	3.4	1.6	1.6
assign	(resid	63	and	name	HN)	(resid	62	and	name	HG)	2.9	1.1	1.1
assign	(resid	63	and	name	HN)	(resid	38	and	name	HB*)	3.9	2.1	2.1
assign	(resid	63	and	name	HN)	(resid	64	and	name	HN)	2.4	0.6	0.6
assign	(resid	63	and	name	HN)	(resid	65	and	name	HB)	3.9	2.1	2.1
assign	(resid	63	and	name	HN)	(resid	65	and	name	HN)	2.9	1.1	1.1

!LYS64

assign	(resid	64	and	name	HA)	(resid	61	and	name	HE*)	3.4	1.6	1.6
assign	(resid	64	and	name	HA)	(resid	65	and	name	HA)	3.4	1.6	1.6
assign	(resid	64	and	name	HA)	(resid	65	and	name	HN)	2.4	0.6	0.6
assign	(resid	64	and	name	HG*)	(resid	65	and	name	HN)	3.9	2.1	2.1
assign	(resid	64	and	name	HN)	(resid	60	and	name	HA)	3.4	1.6	1.6
assign	(resid	64	and	name	HN)	(resid	63	and	name	HA)	2.9	1.1	1.1
assign	(resid	64	and	name	HN)	(resid	63	and	name	HB*)	2.9	1.1	1.1
assign	(resid	64	and	name	HN)	(resid	65	and	name	HB)	3.9	2.1	2.1
assign	(resid	64	and	name	HN)	(resid	65	and	name	HN)	2.4	0.6	0.6

!THR65

assign	(resid	65	and	name	HA)	(resid	40	and	name	HG*)	3.9	2.1	2.1
assign	(resid	65	and	name	HA)	(resid	64	and	name	HG*)	3.4	1.6	1.6
assign	(resid	65	and	name	HA)	(resid	66	and	name	HN)	2.4	0.6	0.6
assign	(resid	65	and	name	HA)	(resid	66	and	name	HG*)	3.4	1.6	1.6
assign	(resid	65	and	name	HB)	(resid	42	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	65	and	name	HG2*)	(resid	41	and	name	HA)	2.9	1.1	1.1
assign	(resid	65	and	name	HG2*)	(resid	42	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	65	and	name	HG2*)	(resid	42	and	name	HN)	3.4	1.6	1.6
assign	(resid	65	and	name	HG2*)	(resid	66	and	name	HB*)	3.4	1.6	1.6
assign	(resid	65	and	name	HN)	(resid	64	and	name	HB*)	3.4	1.6	1.6
assign	(resid	65	and	name	HN)	(resid	66	and	name	HN)	3.9	2.1	2.1

!LYS66

assign	(resid	66	and	name	HN)	(resid	41	and	name	HA)	3.4	1.6	1.6
assign	(resid	66	and	name	HN)	(resid	65	and	name	HB)	3.4	1.6	1.6
assign	(resid	66	and	name	HN)	(resid	65	and	name	HG2*)	2.4	0.6	0.6
assign	(resid	66	and	name	HN)	(resid	67	and	name	HN)	3.9	2.1	2.1

!VAL67

assign	(resid	67	and	name	HA)	(resid	43	and	name	HA)	3.9	2.1	2.1
assign	(resid	67	and	name	HA)	(resid	43	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	67	and	name	HG1*)	(resid	42	and	name	HN)	3.4	1.6	1.6
assign	(resid	67	and	name	HG1*)	(resid	68	and	name	HN)	2.9	1.1	1.1
assign	(resid	67	and	name	HG1*)	(resid	69	and	name	HD2)	3.4	1.6	1.6
assign	(resid	67	and	name	HG1*)	(resid	69	and	name	HE2)	2.9	1.1	1.1
assign	(resid	67	and	name	HG2*)	(resid	42	and	name	HN)	3.9	2.1	2.1
assign	(resid	67	and	name	HG2*)	(resid	57	and	name	HA)	2.9	1.1	1.1
assign	(resid	67	and	name	HG2*)	(resid	60	and	name	HN)	2.9	1.1	1.1
assign	(resid	67	and	name	HG2*)	(resid	66	and	name	HA)	2.9	1.1	1.1
assign	(resid	67	and	name	HN)	(resid	66	and	name	HG*)	3.4	1.6	1.6
assign	(resid	67	and	name	HN)	(resid	66	and	name	HB*)	2.9	1.1	1.1
assign	(resid	67	and	name	HN)	(resid	66	and	name	HA)	2.4	0.6	0.6
assign	(resid	67	and	name	HN)	(resid	68	and	name	HN)	3.9	2.1	2.1

!TYR68

assign	(resid	68	and	name	HB*)	(resid	69	and	name	HN)	3.9	2.1	2.1
assign	(resid	68	and	name	HD2)	(resid	70	and	name	HD1)	2.9	1.1	1.1
assign	(resid	68	and	name	HD1)	(resid	104	and	name	HB*)	3.4	1.6	1.6
assign	(resid	68	and	name	HD1)	(resid	104	and	name	HD*)	2.9	1.1	1.1
assign	(resid	68	and	name	HD1)	(resid	104	and	name	HG)	3.4	1.6	1.6
assign	(resid	68	and	name	HE2)	(resid	43	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	68	and	name	HE2)	(resid	70	and	name	HB1)	2.9	1.1	1.1
assign	(resid	68	and	name	HE2)	(resid	70	and	name	HB2)	3.4	1.6	1.6
assign	(resid	68	and	name	HE2)	(resid	70	and	name	HD1)	2.9	1.1	1.1
assign	(resid	68	and	name	HE1)	(resid	104	and	name	HD*)	2.9	1.1	1.1
assign	(resid	68	and	name	HE1)	(resid	104	and	name	HG)	3.9	2.1	2.1
assign	(resid	68	and	name	HN)	(resid	42	and	name	HN)	3.4	1.6	1.6
assign	(resid	68	and	name	HN)	(resid	44	and	name	HN)	3.4	1.6	1.6
assign	(resid	68	and	name	HN)	(resid	43	and	name	HA)	2.9	1.1	1.1
assign	(resid	68	and	name	HN)	(resid	43	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	68	and	name	HN)	(resid	67	and	name	HA)	2.4	0.6	0.6
assign	(resid	68	and	name	HN)	(resid	67	and	name	HB)	3.9	2.1	2.1
assign	(resid	68	and	name	HN)	(resid	69	and	name	HN)	3.9	2.1	2.1

!TYR69

assign	(resid	69	and	name	HA)	(resid	44	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	69	and	name	HD1)	(resid	53	and	name	HB*)	3.9	2.1	2.1
assign	(resid	69	and	name	HD2)	(resid	67	and	name	HB)	3.9	2.1	2.1
assign	(resid	69	and	name	HD1)	(resid	68	and	name	HA)	3.4	1.6	1.6

assign	(resid	69	and	name	HD2)	(resid	44	and	name	HB)	3.4	1.6	1.6
assign	(resid	69	and	name	HE1)	(resid	53	and	name	HG*)	3.9	2.1	2.1
assign	(resid	69	and	name	HE1)	(resid	53	and	name	HD*)	3.4	1.6	1.6
assign	(resid	69	and	name	HE2)	(resid	67	and	name	HB)	2.4	0.6	0.6
assign	(resid	69	and	name	HE1)	(resid	68	and	name	HA)	3.9	2.1	2.1
assign	(resid	69	and	name	HN)	(resid	68	and	name	HA)	2.4	0.6	0.6
assign	(resid	69	and	name	HN)	(resid	68	and	name	HD1)	3.9	2.1	2.1
assign	(resid	69	and	name	HN)	(resid	68	and	name	HE1)	3.9	2.1	2.1
assign	(resid	69	and	name	HN)	(resid	70	and	name	HA)	3.9	2.1	2.1
assign	(resid	69	and	name	HN)	(resid	70	and	name	HN)	3.9	2.1	2.1

!PHE70

assign	(resid	70	and	name	HD1)	(resid	69	and	name	HA)	3.4	1.6	1.6
assign	(resid	70	and	name	HD2)	(resid	71	and	name	HA)	3.4	1.6	1.6
assign	(resid	70	and	name	HE2)	(resid	76	and	name	HB2)	3.4	1.6	1.6
assign	(resid	70	and	name	HZ)	(resid	76	and	name	HB2)	3.4	1.6	1.6
assign	(resid	70	and	name	HE2)	(resid	76	and	name	HG*)	3.4	1.6	1.6
assign	(resid	70	and	name	HZ)	(resid	76	and	name	HG*)	3.4	1.6	1.6
assign	(resid	70	and	name	HD2)	(resid	72	and	name	HA*)	3.9	2.1	2.1
assign	(resid	70	and	name	HZ)	(resid	77	and	name	HA)	3.4	1.6	1.6
assign	(resid	70	and	name	HZ)	(resid	77	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	70	and	name	HE1)	(resid	77	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	70	and	name	HZ)	(resid	77	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	70	and	name	HE1)	(resid	77	and	name	HG)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	44	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	44	and	name	HN)	3.9	2.1	2.1
assign	(resid	70	and	name	HN)	(resid	69	and	name	HA)	2.4	0.6	0.6
assign	(resid	70	and	name	HN)	(resid	69	and	name	HB1)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	69	and	name	HB2)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	69	and	name	HD2)	3.4	1.6	1.6
assign	(resid	70	and	name	HN)	(resid	71	and	name	HN)	3.9	2.1	2.1

!GLN71

assign	(resid	71	and	name	HA)	(resid	46	and	name	HA)	3.4	1.6	1.6
assign	(resid	71	and	name	HA)	(resid	46	and	name	HB*)	3.9	2.1	2.1
assign	(resid	71	and	name	HA)	(resid	72	and	name	HN)	2.9	1.1	1.1
assign	(resid	71	and	name	HN)	(resid	46	and	name	HB*)	3.9	2.1	2.1
assign	(resid	71	and	name	HN)	(resid	70	and	name	HA)	2.4	0.6	0.6
assign	(resid	71	and	name	HN)	(resid	70	and	name	HB1)	3.9	2.1	2.1
assign	(resid	71	and	name	HN)	(resid	70	and	name	HB2)	3.9	2.1	2.1
assign	(resid	71	and	name	HN)	(resid	70	and	name	HD2)	2.9	1.1	1.1
assign	(resid	71	and	name	HN)	(resid	72	and	name	HN)	2.9	1.1	1.1

!GLY72

assign	(resid	72	and	name	HA*)	(resid	71	and	name	HA)	3.4	1.6	1.6
assign	(resid	72	and	name	HN)	(resid	46	and	name	HB*)	2.9	1.1	1.1
assign	(resid	72	and	name	HN)	(resid	46	and	name	HN)	3.4	1.6	1.6
assign	(resid	72	and	name	HN)	(resid	·70	and	name	HD2)	2.9	1.1	1.1
assign	(resid	72	and	name	HN)	(resid	71	and	name	HG*)	2.9	1.1	1.1
assign	(resid	72	and	name	HN)	(resid	73	and	name	HN)	2.9	1.1	1.1
assign	(resid	72	and	name	HN)	(resid	74	and	name	HN)	3.9	2.1	2.1
assign	(resid	72	and	name	HN)	(resid	75	and	name	HN)	3.9	2.1	2.1

!GLY73

assign	(resid	73	and	name	HA*)	(resid	74	and	name	HN)	3.9	2.1	2.1
assign	(resid	73	and	name	HN)	(resid	72	and	name	HA*)	2.9	1.1	1.1
assign	(resid	73	and	name	HN)	(resid	74	and	name	HN)	3.4	1.6	1.6
assign	(resid	73	and	name	HN)	(resid	75	and	name	HN)	3.9	2.1	2.1

!ASN74

assign	(resid	74	and	name	HN)	(resid	75	and	name	HN)	2.9	1.1	1.1

!ASN75

assign	(resid	75	and	name	HA)	(resid	76	and	name	HN)	3.9	2.1	2.1
assign	(resid	75	and	name	HB*)	(resid	76	and	name	HN)	2.9	1.1	1.1
assign	(resid	75	and	name	HN)	(resid	74	and	name	HA)	3.4	1.6	1.6
assign	(resid	75	and	name	HN)	(resid	74	and	name	HB*)	3.9	2.1	2.1
assign	(resid	75	and	name	HN)	(resid	76	and	name	HA)	3.9	2.1	2.1
assign	(resid	75	and	name	HN)	(resid	76	and	name	HG*)	3.9	2.1	2.1
assign	(resid	75	and	name	HN)	(resid	76	and	name	HN)	2.4	0.6	0.6

!GLU76

assign	(resid	76	and	name	HA)	(resid	79	and	name	HB)	2.9	1.1	1.1
assign	(resid	76	and	name	HG*)	(resid	77	and	name	HN)	3.4	1.6	1.6
assign	(resid	76	and	name	HN)	(resid	70	and	name	HZ)	2.9	1.1	1.1
assign	(resid	76	and	name	HN)	(resid	72	and	name	HA*)	3.9	2.1	2.1
assign	(resid	76	and	name	HN)	(resid	73	and	name	HN)	3.4	1.6	1.6
assign	(resid	76	and	name	HN)	(resid	77	and	name	HB*)	3.9	2.1	2.1
assign	(resid	76	and	name	HN)	(resid	77	and	name	HN)	2.9	1.1	1.1

!LEU77

assign	(resid	77	and	name	HA)	(resid	45	and	name	HB*)	3.9	2.1	2.1
assign	(resid	77	and	name	HA)	(resid	80	and	name	HB*)	2.9	1.1	1.1
assign	(resid	77	and	name	HD1*)	(resid	80	and	name	HB*)	2.9	1.1	1.1
assign	(resid	77	and	name	HD1*)	(resid	90	and	name	HN)	3.4	1.6	1.6

assign	(resid	77	and	name	HD2*)	(resid	70	and	name	HD2)	3.9	2.1	2.1
assign	(resid	77	and	name	HD2*)	(resid	81	and	name	HN)	3.9	2.1	2.1
assign	(resid	77	and	name	HN)	(resid	70	and	name	HE2)	2.9	1.1	1.1
assign	(resid	77	and	name	HN)	(resid	74	and	name	HA)	3.4	1.6	1.6
assign	(resid	77	and	name	HN)	(resid	75	and	name	HN)	3.9	2.1	2.1
assign	(resid	77	and	name	HN)	(resid	76	and	name	HA)	3.4	1.6	1.6
assign	(resid	77	and	name	HN)	(resid	78	and	name	HN)	2.9	1.1	1.1
assign	(resid	77	and	name	HN)	(resid	79	and	name	HN)	3.4	1.6	1.6

!GLY78

assign	(resid	78	and	name	HA*)	(resid	79	and	name	HN)	2.9	1.1	1.1
assign	(resid	78	and	name	HN)	(resid	74	and	name	HA)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	75	and	name	HA)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	76	and	name	HN)	3.4	1.6	1.6
assign	(resid	78	and	name	HN)	(resid	77	and	name	HB*)	2.9	1.1	1.1
assign	(resid	78	and	name	HN)	(resid	77	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	78	and	name	HN)	(resid	77	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	77	and	name	HG)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	79	and	name	HB)	3.4	1.6	1.6
assign	(resid	78	and	name	HN)	(resid	79	and	name	HA)	3.9	2.1	2.1
assign	(resid	78	and	name	HN)	(resid	79	and	name	HN)	2.4	0.6	0.6
assign	(resid	78	and	name	HN)	(resid	80	and	name	HN)	2.9	1.1	1.1

!THR79

assign	(resid	79	and	name	HA)	(resid	80	and	name	HN)	2.9	1.1	1.1
assign	(resid	79	and	name	HA)	(resid	82	and	name	HA*)	3.9	2.1	2.1
assign	(resid	79	and	name	HB)	(resid	76	and	name	HB*)	3.4	1.6	1.6
assign	(resid	79	and	name	HB)	(resid	80	and	name	HB*)	3.9	2.1	2.1
assign	(resid	79	and	name	HB)	(resid	80	and	name	HN)	3.4	1.6	1.6
assign	(resid	79	and	name	HG2*)	(resid	80	and	name	HN)	2.9	1.1	1.1
assign	(resid	79	and	name	HN)	(resid	76	and	name	HA)	3.9	2.1	2.1
assign	(resid	79	and	name	HN)	(resid	76	and	name	HN)	3.4	1.6	1.6
assign	(resid	79	and	name	HN)	(resid	77	and	name	HA)	3.9	2.1	2.1
assign	(resid	79	and	name	HN)	(resid	80	and	name	HN)	2.9	1.1	1.1
assign	(resid	79	and	name	HN)	(resid	81	and	name	HN)	3.4	1.6	1.6

!ALA80

assign	(resid	80	and	name	HA)	(resid	79	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	80	and	name	HA)	(resid	81	and	name	HN)	2.9	1.1	1.1
assign	(resid	80	and	name	HB*)	(resid	77	and	name	HG)	3.4	1.6	1.6
assign	(resid	80	and	name	HB*)	(resid	14	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	80	and	name	HB*)	(resid	81	and	name	HN)	2.9	1.1	1.1
assign	(resid	80	and	name	HN)	(resid	77	and	name	HA)	3.4	1.6	1.6
assign	(resid	80	and	name	HN)	(resid	77	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	80	and	name	HN)	(resid	81	and	name	HN)	2.9	1.1	1.1
assign	(resid	80	and	name	HN)	(resid	11	and	name	HB*)	3.9	2.1	2.1

!VAL81

assign	(resid	81	and	name	HB)	(resid	18	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	81	and	name	HG1*)	(resid	18	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	81	and	name	HG1*)	(resid	18	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	81	and	name	HG1*)	(resid	77	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	81	and	name	HG1*)	(resid	80	and	name	HB*)	3.4	1.6	1.6
assign	(resid	81	and	name	HG1*)	(resid	82	and	name	HN)	3.4	1.6	1.6
assign	(resid	81	and	name	HG1*)	(resid	83	and	name	HN)	3.9	2.1	2.1
assign	(resid	81	and	name	HG1*)	(resid	90	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	81	and	name	HG2*)	(resid	80	and	name	HB*)	3.4	1.6	1.6
assign	(resid	81	and	name	HG2*)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	81	and	name	HG2*)	(resid	82	and	name	HN)	2.9	1.1	1.1
assign	(resid	81	and	name	HG2*)	(resid	83	and	name	HN)	3.4	1.6	1.6
assign	(resid	81	and	name	HG2*)	(resid	78	and	name	HA2)	3.4	1.6	1.6
assign	(resid	81	and	name	HN)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	77	and	name	HA)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	77	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	25	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	81	and	name	HN)	(resid	78	and	name	HA2)	3.4	1.6	1.6
assign	(resid	81	and	name	HN)	(resid	78	and	name	HN)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	79	and	name	HA)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	79	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	81	and	name	HN)	(resid	82	and	name	HA*)	3.4	1.6	1.6
assign	(resid	81	and	name	HN)	(resid	82	and	name	HN)	2.4	0.6	0.6
assign	(resid	81	and	name	HN)	(resid	83	and	name	HN)	3.4	1.6	1.6

!GLY82

assign	(resid	82	and	name	HN)	(resid	79	and	name	HA)	2.9	1.1	1.1
assign	(resid	82	and	name	HN)	(resid	79	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	82	and	name	HN)	(resid	79	and	name	HB)	3.9	2.1	2.1
assign	(resid	82	and	name	HN)	(resid	80	and	name	HN)	3.9	2.1	2.1
assign	(resid	82	and	name	HN)	(resid	81	and	name	HA)	2.9	1.1	1.1
assign	(resid	82	and	name	HN)	(resid	81	and	name	HB)	3.9	2.1	2.1
assign	(resid	82	and	name	HN)	(resid	83	and	name	HN)	2.9	1.1	1.1

!LYS83

assign	(resid	83	and	name	HA	)	(resid	84	and	name	HD*	)	3.9	2.1	2.1
assign	(resid	83	and	name	HA	)	(resid	84	and	name	HN	)	3.9	2.1	2.1
assign	(resid	83	and	name	HA	)	(resid	85	and	name	HN	)	3.9	2.1	2.1
assign	(resid	83	and	name	HB*	)	(resid	85	and	name	HZ	)	3.4	1.6	1.6
assign	(resid	83	and	name	HB*	)	(resid	85	and	name	HE2	)	3.4	1.6	1.6
assign	(resid	83	and	name	HB*	)	(resid	85	and	name	HD2	)	3.4	1.6	1.6
assign	(resid	83	and	name	HN	)	(resid	79	and	name	HA	)	2.9	1.1	1.1
assign	(resid	83	and	name	HN	)	(resid	82	and	name	HA*	)	2.9	1.1	1.1
assign	(resid	83	and	name	HN	)	(resid	84	and	name	HA	)	3.9	2.1	2.1
assign	(resid	83	and	name	HN	)	(resid	84	and	name	HN	)	3.4	1.6	1.6

!LEU84

assign	(resid	84	and	name	HA	)	(resid	85	and	name	HN	)	3.9	2.1	2.1
assign	(resid	84	and	name	HN	)	(resid	83	and	name	HB*	)	3.4	1.6	1.6
assign	(resid	84	and	name	HN	)	(resid	83	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	84	and	name	HN	)	(resid	85	and	name	HE2	)	3.4	1.6	1.6
assign	(resid	84	and	name	HN	)	(resid	85	and	name	HD2	)	3.4	1.6	1.6
assign	(resid	84	and	name	HN	)	(resid	85	and	name	HN	)	2.9	1.1	1.1

!PHE85

assign	(resid	85	and	name	HA	)	(resid	84	and	name	HD*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HD1	)	(resid	87	and	name	HG1*	)	2.4	0.6	0.6
assign	(resid	85	and	name	HD1	)	(resid	25	and	name	HD1*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HD1	)	(resid	87	and	name	HB	)	3.9	2.1	2.1
assign	(resid	85	and	name	HD2	)	(resid	83	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HE2	)	(resid	83	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HE2	)	(resid	83	and	name	HD*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HE2	)	(resid	83	and	name	HE*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HE1	)	(resid	25	and	name	HD1*	)	2.9	1.1	1.1
assign	(resid	85	and	name	HE1	)	(resid	87	and	name	HG1*	)	2.9	1.1	1.1
assign	(resid	85	and	name	HE1	)	(resid	87	and	name	HB	)	3.4	1.6	1.6
assign	(resid	85	and	name	HZ	)	(resid	83	and	name	HG*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HZ	)	(resid	83	and	name	HD*	)	3.9	2.1	2.1
assign	(resid	85	and	name	HZ	)	(resid	83	and	name	HE*	)	2.9	1.1	1.1
assign	(resid	85	and	name	HZ	)	(resid	25	and	name	HD1*	)	2.4	0.6	0.6
assign	(resid	85	and	name	HZ	)	(resid	87	and	name	HG1*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HZ	)	(resid	87	and	name	HB	)	3.4	1.6	1.6
assign	(resid	85	and	name	HN	)	(resid	83	and	name	HB*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HN	)	(resid	83	and	name	HG*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HN	)	(resid	84	and	name	HB*	)	2.9	1.1	1.1
assign	(resid	85	and	name	HN	)	(resid	84	and	name	HG	)	2.9	1.1	1.1
assign	(resid	85	and	name	HN	)	(resid	84	and	name	HD*	)	3.4	1.6	1.6
assign	(resid	85	and	name	HN	)	(resid	86	and	name	HN	)	3.4	1.6	1.6

!ARG86

assign	(resid	86	and	name	HA	)	(resid	87	and	name	HN	)	3.9	2.1	2.1
assign	(resid	86	and	name	HE	)	(resid	85	and	name	HA	)	3.9	2.1	2.1
assign	(resid	86	and	name	HG*	)	(resid	87	and	name	HN	)	3.9	2.1	2.1
assign	(resid	86	and	name	HN	)	(resid	85	and	name	HA	)	2.9	1.1	1.1
assign	(resid	86	and	name	HN	)	(resid	85	and	name	HB2	)	2.9	1.1	1.1
assign	(resid	86	and	name	HN	)	(resid	85	and	name	HB1	)	2.9	1.1	1.1
assign	(resid	86	and	name	HN	)	(resid	85	and	name	HD1	)	2.9	1.1	1.1
assign	(resid	86	and	name	HN	)	(resid	85	and	name	HE1	)	3.9	2.1	2.1
assign	(resid	86	and	name	HN	)	(resid	87	and	name	HN	)	2.9	1.1	1.1

!VAL87

assign	(resid	87	and	name	HG1*	)	(resid	86	and	name	HA	)	3.4	1.6	1.6
assign	(resid	87	and	name	HG1*	)	(resid	85	and	name	HD1	)	2.9	1.1	1.1
assign	(resid	87	and	name	HG1*	)	(resid	85	and	name	HE1	)	3.4	1.6	1.6
assign	(resid	87	and	name	HN	)	(resid	86	and	name	HB*	)	2.9	1.1	1.1
assign	(resid	87	and	name	HN	)	(resid	86	and	name	HD*	)	3.4	1.6	1.6
assign	(resid	87	and	name	HN	)	(resid	88	and	name	HA*	)	3.9	2.1	2.1
assign	(resid	87	and	name	HN	)	(resid	88	and	name	HN	)	2.9	1.1	1.1

!GLY88

assign	(resid	88	and	name	HA*	)	(resid	89	and	name	HN	)	2.9	1.1	1.1
assign	(resid	88	and	name	HN	)	(resid	87	and	name	HG1*	)	2.4	0.6	0.6
assign	(resid	88	and	name	HN	)	(resid	87	and	name	HG2*	)	2.9	1.1	1.1
assign	(resid	88	and	name	HN	)	(resid	87	and	name	HB	)	3.9	2.1	2.1
assign	(resid	88	and	name	HN	)	(resid	89	and	name	HN	)	2.9	1.1	1.1

!VAL89

assign	(resid	89	and	name	HA	)	(resid	45	and	name	HN	)	2.9	1.1	1.1
assign	(resid	89	and	name	HA	)	(resid	44	and	name	HB	)	3.4	1.6	1.6
assign	(resid	89	and	name	HA	)	(resid	90	and	name	HN	)	2.4	0.6	0.6
assign	(resid	89	and	name	HG1*	)	(resid	27	and	name	HN	)	3.9	2.1	2.1
assign	(resid	89	and	name	HG1*	)	(resid	27	and	name	HA	)	2.9	1.1	1.1
assign	(resid	89	and	name	HG1*	)	(resid	26	and	name	HA*	)	3.9	2.1	2.1
assign	(resid	89	and	name	HG1*	)	(resid	26	and	name	HN	)	2.9	1.1	1.1
assign	(resid	89	and	name	HG2*	)	(resid	30	and	name	HG2*	)	3.4	1.6	1.6
assign	(resid	89	and	name	HG2*	)	(resid	44	and	name	HB	)	2.9	1.1	1.1
assign	(resid	89	and	name	HG2*	)	(resid	44	and	name	HA	)	3.9	2.1	2.1
assign	(resid	89	and	name	HG2*	)	(resid	90	and	name	HN	)	2.9	1.1	1.1

assign	(resid	89	and	name	HN)	(resid	25	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	89	and	name	HN)	(resid	87	and	name	HB)	3.4	1.6	1.6
assign	(resid	89	and	name	HN)	(resid	87	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	89	and	name	HN)	(resid	90	and	name	HN)	3.9	2.1	2.1

!VAL90

assign	(resid	90	and	name	HG1*)	(resid	18	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	90	and	name	HG1*)	(resid	91	and	name	HN)	2.9	1.1	1.1
assign	(resid	90	and	name	HG1*)	(resid	23	and	name	HD2)	3.4	1.6	1.6
assign	(resid	90	and	name	HG1*)	(resid	23	and	name	HB*)	3.4	1.6	1.6
assign	(resid	90	and	name	HG1*)	(resid	77	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	90	and	name	HG1*)	(resid	77	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	90	and	name	HG1*)	(resid	14	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	90	and	name	HG1*)	(resid	14	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	90	and	name	HG2*)	(resid	77	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	90	and	name	HG2*)	(resid	77	and	name	HG)	2.9	1.1	1.1
assign	(resid	90	and	name	HG2*)	(resid	77	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	90	and	name	HG2*)	(resid	44	and	name	HA)	3.4	1.6	1.6
assign	(resid	90	and	name	HN)	(resid	44	and	name	HB)	3.4	1.6	1.6
assign	(resid	90	and	name	HN)	(resid	44	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	90	and	name	HN)	(resid	89	and	name	HB)	3.4	1.6	1.6

!SER91

assign	(resid	91	and	name	HA)	(resid	43	and	name	HB)	3.9	2.1	2.1
assign	(resid	91	and	name	HB1)	(resid	34	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	91	and	name	HB1)	(resid	34	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	91	and	name	HB1)	(resid	92	and	name	HA)	3.4	1.6	1.6
assign	(resid	91	and	name	HN)	(resid	24	and	name	HA)	3.9	2.1	2.1
assign	(resid	91	and	name	HN)	(resid	34	and	name	HD*)	3.4	1.6	1.6
assign	(resid	91	and	name	HN)	(resid	90	and	name	HA)	2.4	0.6	0.6

!ILE92

assign	(resid	92	and	name	HA)	(resid	23	and	name	HB*)	3.9	2.1	2.1
assign	(resid	92	and	name	HD1*)	(resid	14	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	92	and	name	HD1*)	(resid	14	and	name	HG*)	3.9	2.1	2.1
assign	(resid	92	and	name	HD1*)	(resid	18	and	name	HA)	3.4	1.6	1.6
assign	(resid	92	and	name	HD1*)	(resid	18	and	name	HN)	3.4	1.6	1.6
assign	(resid	92	and	name	HD1*)	(resid	18	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	92	and	name	HD1*)	(resid	18	and	name	HG1*)	2.9	1.1	1.1
assign	(resid	92	and	name	HD1*)	(resid	23	and	name	HB*)	2.9	1.1	1.1
assign	(resid	92	and	name	HD1*)	(resid	93	and	name	HN)	3.9	2.1	2.1
assign	(resid	92	and	name	HG2*)	(resid	100	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	92	and	name	HG2*)	(resid	41	and	name	HN)	3.9	2.1	2.1
assign	(resid	92	and	name	HG2*)	(resid	40	and	name	HB*)	3.4	1.6	1.6
assign	(resid	92	and	name	HG2*)	(resid	93	and	name	HN)	2.9	1.1	1.1
assign	(resid	92	and	name	HG2*)	(resid	94	and	name	HN)	3.4	1.6	1.6
assign	(resid	92	and	name	HG2*)	(resid	95	and	name	HA)	3.9	2.1	2.1
assign	(resid	92	and	name	HN)	(resid	42	and	name	HA)	3.4	1.6	1.6
assign	(resid	92	and	name	HN)	(resid	91	and	name	HA)	2.4	0.6	0.6
assign	(resid	92	and	name	HN)	(resid	91	and	name	HB1)	3.9	2.1	2.1
assign	(resid	92	and	name	HN)	(resid	91	and	name	HB2)	3.4	1.6	1.6

!LEU93

assign	(resid	93	and	name	HB*)	(resid	94	and	name	HB*)	3.9	2.1	2.1
assign	(resid	93	and	name	HB*)	(resid	94	and	name	HN)	2.9	1.1	1.1
assign	(resid	93	and	name	HD1*)	(resid	22	and	name	HE*)	3.9	2.1	2.1
assign	(resid	93	and	name	HD1*)	(resid	24	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	93	and	name	HD1*)	(resid	23	and	name	HA)	3.4	1.6	1.6
assign	(resid	93	and	name	HD1*)	(resid	23	and	name	HN)	3.9	2.1	2.1
assign	(resid	93	and	name	HD1*)	(resid	37	and	name	HA*)	3.4	1.6	1.6
assign	(resid	93	and	name	HD1*)	(resid	92	and	name	HA)	3.4	1.6	1.6
assign	(resid	93	and	name	HD1*)	(resid	94	and	name	HN)	3.4	1.6	1.6
assign	(resid	93	and	name	HD2*)	(resid	94	and	name	HN)	3.4	1.6	1.6
assign	(resid	93	and	name	HD2*)	(resid	38	and	name	HB*)	3.9	2.1	2.1
assign	(resid	93	and	name	HG)	(resid	92	and	name	HA)	3.9	2.1	2.1
assign	(resid	93	and	name	HG)	(resid	94	and	name	HN)	3.4	1.6	1.6
assign	(resid	93	and	name	HN)	(resid	23	and	name	HB*)	3.9	2.1	2.1
assign	(resid	93	and	name	HN)	(resid	24	and	name	HN)	3.9	2.1	2.1
assign	(resid	93	and	name	HN)	(resid	92	and	name	HA)	2.4	0.6	0.6
assign	(resid	93	and	name	HN)	(resid	92	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	93	and	name	HN)	(resid	94	and	name	HA)	3.9	2.1	2.1
assign	(resid	93	and	name	HN)	(resid	94	and	name	HN)	2.9	1.1	1.1

!GLU94

assign	(resid	94	and	name	HG*)	(resid	22	and	name	HD*)	3.4	1.6	1.6
assign	(resid	94	and	name	HG*)	(resid	22	and	name	HE*)	3.9	2.1	2.1
assign	(resid	94	and	name	HB1)	(resid	95	and	name	HN)	3.4	1.6	1.6
assign	(resid	94	and	name	HB2)	(resid	95	and	name	HN)	2.9	1.1	1.1
assign	(resid	94	and	name	HB2)	(resid	96	and	name	HN)	3.4	1.6	1.6
assign	(resid	94	and	name	HN)	(resid	21	and	name	HA*)	3.9	2.1	2.1
assign	(resid	94	and	name	HN)	(resid	22	and	name	HA)	3.9	2.1	2.1
assign	(resid	94	and	name	HN)	(resid	23	and	name	HA)	3.9	2.1	2.1

assign	(resid	94	and	name	HN)	(resid	93	and	name	HA)	3.9	2.1	2.1
assign	(resid	94	and	name	HN)	(resid	95	and	name	HA)	3.9	2.1	2.1
assign	(resid	94	and	name	HN)	(resid	95	and	name	HN)	3.4	1.6	1.6

!ALA95

assign	(resid	95	and	name	HA)	(resid	96	and	name	HA*)	3.9	2.1	2.1
assign	(resid	95	and	name	HA)	(resid	96	and	name	HN)	2.9	1.1	1.1
assign	(resid	95	and	name	HB*)	(resid	94	and	name	HA)	3.4	1.6	1.6
assign	(resid	95	and	name	HB*)	(resid	96	and	name	HA*)	3.9	2.1	2.1
assign	(resid	95	and	name	HB*)	(resid	96	and	name	HN)	3.4	1.6	1.6
assign	(resid	95	and	name	HB*)	(resid	101	and	name	HB*)	3.9	2.1	2.1
assign	(resid	95	and	name	HB*)	(resid	101	and	name	HD2*)	2.9	1.1	1.1
assign	(resid	95	and	name	HB*)	(resid	101	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	95	and	name	HN)	(resid	94	and	name	HA)	2.4	0.6	0.6
assign	(resid	95	and	name	HN)	(resid	94	and	name	HG*)	3.4	1.6	1.6
assign	(resid	95	and	name	HN)	(resid	96	and	name	HA*)	3.9	2.1	2.1
assign	(resid	95	and	name	HN)	(resid	96	and	name	HN)	2.9	1.1	1.1
assign	(resid	95	and	name	HN)	(resid	17	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	95	and	name	HN)	(resid	17	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	95	and	name	HN)	(resid	101	and	name	HG*)	3.9	2.1	2.1

!GLY96

assign	(resid	96	and	name	HA*)	(resid	94	and	name	HA)	3.9	2.1	2.1
assign	(resid	96	and	name	HA*)	(resid	97	and	name	HN)	3.4	1.6	1.6
assign	(resid	96	and	name	HN)	(resid	21	and	name	HA#)	2.9	1.1	1.1
assign	(resid	96	and	name	HN)	(resid	97	and	name	HN)	2.9	1.1	1.1
assign	(resid	96	and	name	HN)	(resid	17	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	96	and	name	HN)	(resid	17	and	name	HG2*)	3.9	2.1	2.1

!ASP97

assign	(resid	97	and	name	HA)	(resid	16	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	97	and	name	HA)	(resid	16	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	97	and	name	HN)	(resid	16	and	name	HD1*)	3.9	2.1	2.1
assign	(resid	97	and	name	HN)	(resid	16	and	name	HD2*)	3.9	2.1	2.1
assign	(resid	97	and	name	HN)	(resid	20	and	name	HA)	3.9	2.1	2.1
assign	(resid	97	and	name	HN)	(resid	98	and	name	HN)	2.9	1.1	1.1

!SER98

assign	(resid	98	and	name	HA)	(resid	16	and	name	HD1*)	2.9	1.1	1.1
assign	(resid	98	and	name	HA)	(resid	16	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	98	and	name	HB*)	(resid	16	and	name	HB2)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	16	and	name	HB1)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	16	and	name	HB2)	3.9	2.1	2.1
assign	(resid	98	and	name	HN)	(resid	16	and	name	HD2*)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	16	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	95	and	name	HA)	3.9	2.1	2.1
assign	(resid	98	and	name	HN)	(resid	95	and	name	HB*)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	96	and	name	HA*)	2.9	1.1	1.1
assign	(resid	98	and	name	HN)	(resid	97	and	name	HA)	2.9	1.1	1.1
assign	(resid	98	and	name	HN)	(resid	97	and	name	HB2)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	97	and	name	HB1)	3.4	1.6	1.6
assign	(resid	98	and	name	HN)	(resid	99	and	name	HA)	3.9	2.1	2.1
assign	(resid	98	and	name	HN)	(resid	99	and	name	HN)	3.4	1.6	1.6

!ASP99

assign	(resid	99	and	name	HA)	(resid	102	and	name	HB)	3.9	2.1	2.1
assign	(resid	99	and	name	HA)	(resid	102	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	99	and	name	HB1)	(resid	102	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	99	and	name	HB2)	(resid	102	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	99	and	name	HN)	(resid	98	and	name	HA)	2.9	1.1	1.1
assign	(resid	99	and	name	HN)	(resid	98	and	name	HB*)	3.4	1.6	1.6
assign	(resid	99	and	name	HN)	(resid	100	and	name	HN)	2.9	1.1	1.1

!ILE100

assign	(resid	100	and	name	HA)	(resid	101	and	name	HB*)	3.9	2.1	2.1
assign	(resid	100	and	name	HG2*)	(resid	14	and	name	HA)	3.9	2.1	2.1
assign	(resid	100	and	name	HG2*)	(resid	13	and	name	HB*)	3.4	1.6	1.6
assign	(resid	100	and	name	HN)	(resid	98	and	name	HA)	3.4	1.6	1.6
assign	(resid	100	and	name	HN)	(resid	99	and	name	HA)	3.4	1.6	1.6
assign	(resid	100	and	name	HN)	(resid	99	and	name	HB*)	3.9	2.1	2.1
assign	(resid	100	and	name	HN)	(resid	101	and	name	HN)	3.4	1.6	1.6

!LEU101

assign	(resid	101	and	name	HA)	(resid	102	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	101	and	name	HD1*)	(resid	102	and	name	HN)	3.9	2.1	2.1
assign	(resid	101	and	name	HD2*)	(resid	102	and	name	HN)	3.4	1.6	1.6
assign	(resid	101	and	name	HA)	(resid	104	and	name	HD*)	3.9	2.1	2.1
assign	(resid	101	and	name	HN)	(resid	100	and	name	HA)	3.4	1.6	1.6
assign	(resid	101	and	name	HN)	(resid	100	and	name	HB)	3.4	1.6	1.6
assign	(resid	101	and	name	HN)	(resid	102	and	name	HG2*)	2.9	1.1	1.1
assign	(resid	101	and	name	HN)	(resid	102	and	name	HN)	2.9	1.1	1.1
assign	(resid	101	and	name	HN)	(resid	104	and	name	HN)	3.4	1.6	1.6

!THR102

assign	(resid	102	and	name	HG2*)	(resid	99	and	name	HA)	3.4	1.6	1.6

assign	(resid	102	and	name	HB)	(resid	103	and	name	HN)	3.9	2.1	2.1
assign	(resid	102	and	name	HN)	(resid	99	and	name	HA)	3.9	2.1	2.1
assign	(resid	102	and	name	HN)	(resid	99	and	name	HB*)	3.9	2.1	2.1
assign	(resid	102	and	name	HN)	(resid	101	and	name	HA)	3.4	1.6	1.6
assign	(resid	102	and	name	HN)	(resid	101	and	name	HB*)	3.4	1.6	1.6
assign	(resid	102	and	name	HN)	(resid	101	and	name	HG)	3.4	1.6	1.6
assign	(resid	102	and	name	HN)	(resid	103	and	name	HN)	3.4	1.6	1.6

!THR103

assign	(resid	103	and	name	HG2*)	(resid	99	and	name	HB*)	3.4	1.6	1.6
assign	(resid	103	and	name	HG2*)	(resid	100	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	103	and	name	HN)	(resid	99	and	name	HA)	3.9	2.1	2.1
assign	(resid	103	and	name	HN)	(resid	99	and	name	HB*)	3.9	2.1	2.1
assign	(resid	103	and	name	HN)	(resid	100	and	name	HA)	3.9	2.1	2.1
assign	(resid	103	and	name	HN)	(resid	100	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	103	and	name	HN)	(resid	100	and	name	HG1*)	3.4	1.6	1.6
assign	(resid	103	and	name	HN)	(resid	100	and	name	HN)	3.9	2.1	2.1
assign	(resid	103	and	name	HN)	(resid	104	and	name	HN)	2.9	1.1	1.1

!LEU104

assign	(resid	104	and	name	HB*)	(resid	105	and	name	HN)	3.9	2.1	2.1
assign	(resid	104	and	name	HD*)	(resid	41	and	name	HD1*)	2.4	0.6	0.6
assign	(resid	104	and	name	HD*)	(resid	41	and	name	HG)	2.9	1.1	1.1
assign	(resid	104	and	name	HD*)	(resid	105	and	name	HN)	3.4	1.6	1.6
assign	(resid	104	and	name	HN)	(resid	100	and	name	HA)	3.9	2.1	2.1
assign	(resid	104	and	name	HN)	(resid	100	and	name	HN)	3.9	2.1	2.1
assign	(resid	104	and	name	HN)	(resid	101	and	name	HA)	2.9	1.1	1.1
assign	(resid	104	and	name	HN)	(resid	103	and	name	HA)	2.9	1.1	1.1
assign	(resid	104	and	name	HN)	(resid	103	and	name	HB)	2.9	1.1	1.1
assign	(resid	104	and	name	HN)	(resid	103	and	name	HG2*)	2.9	1.1	1.1

!ALA105

assign	(resid	105	and	name	HA)	(resid	104	and	name	HB*)	3.4	1.6	1.6
assign	(resid	105	and	name	HA)	(resid	104	and	name	HD*)	3.9	2.1	2.1
assign	(resid	105	and	name	HA)	(resid	104	and	name	HG)	2.9	1.1	1.1
assign	(resid	105	and	name	HB*)	(resid	104	and	name	HA)	3.9	2.1	2.1
assign	(resid	105	and	name	HB*)	(resid	104	and	name	HG)	2.9	1.1	1.1
assign	(resid	105	and	name	HN)	(resid	68	and	name	HE1)	3.9	2.1	2.1
assign	(resid	105	and	name	HN)	(resid	68	and	name	HD1)	3.9	2.1	2.1
assign	(resid	105	and	name	HN)	(resid	101	and	name	HA)	3.4	1.6	1.6
assign	(resid	105	and	name	HN)	(resid	104	and	name	HA)	2.9	1.1	1.1
assign	(resid	105	and	name	HN)	(resid	104	and	name	HG)	2.9	1.1	1.1
assign	(resid	105	and	name	HN)	(resid	104	and	name	HN)	2.9	1.1	1.1

set echo = on end
set message = on end

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!hydrogen bonding restraints, L30P_hbond.tbl, 7/10/98
set echo = off end
set message = off end
!b-sheet hydrogen bonds retraints
assign   (resid   22   and   name   O  )   (resid   93   and   name   HN  )   1.95   0.25   0.25
assign   (resid   22   and   name   O  )   (resid   93   and   name   N   )   2.95   0.25   0.25
assign   (resid   91   and   name   O  )   (resid   24   and   name   HN  )   1.95   0.25   0.25
assign   (resid   91   and   name   O  )   (resid   24   and   name   N   )   2.95   0.25   0.25
assign   (resid   24   and   name   O  )   (resid   91   and   name   HN  )   1.95   0.25   0.25
assign   (resid   24   and   name   O  )   (resid   91   and   name   N   )   2.95   0.25   0.25
assign   (resid   92   and   name   O  )   (resid   41   and   name   HN  )   1.95   0.25   0.25
assign   (resid   92   and   name   O  )   (resid   41   and   name   N   )   2.95   0.25   0.25
assign   (resid   41   and   name   O  )   (resid   92   and   name   HN  )   1.95   0.25   0.25
assign   (resid   41   and   name   O  )   (resid   92   and   name   N   )   2.95   0.25   0.25
assign   (resid   90   and   name   O  )   (resid   43   and   name   HN  )   1.95   0.25   0.25
assign   (resid   90   and   name   O  )   (resid   43   and   name   N   )   2.95   0.25   0.25
assign   (resid   43   and   name   O  )   (resid   90   and   name   HN  )   1.95   0.25   0.25
assign   (resid   43   and   name   O  )   (resid   90   and   name   N   )   2.95   0.25   0.25
assign   (resid   88   and   name   O  )   (resid   45   and   name   HN  )   1.95   0.25   0.25
assign   (resid   88   and   name   O  )   (resid   45   and   name   N   )   2.95   0.25   0.25
assign   (resid   66   and   name   O  )   (resid   42   and   name   HN  )   1.95   0.25   0.25
assign   (resid   66   and   name   O  )   (resid   42   and   name   N   )   2.95   0.25   0.25
assign   (resid   42   and   name   O  )   (resid   68   and   name   HN  )   1.95   0.25   0.25
assign   (resid   42   and   name   O  )   (resid   68   and   name   N   )   2.95   0.25   0.25
assign   (resid   68   and   name   O  )   (resid   44   and   name   HN  )   1.95   0.25   0.25
assign   (resid   68   and   name   O  )   (resid   44   and   name   N   )   2.95   0.25   0.25
assign   (resid   44   and   name   O  )   (resid   70   and   name   HN  )   1.95   0.25   0.25
assign   (resid   44   and   name   O  )   (resid   70   and   name   N   )   2.95   0.25   0.25

!a-Hellix hydrogen bonds restraints
assign   (resid   10   and   name   O  )   (resid   14   and   name   HN  )   1.95   0.25   0.25
assign   (resid   10   and   name   O  )   (resid   14   and   name   N   )   2.95   0.25   0.25
assign   (resid   11   and   name   O  )   (resid   15   and   name   HN  )   1.95   0.25   0.25
assign   (resid   11   and   name   O  )   (resid   15   and   name   N   )   2.95   0.25   0.25
assign   (resid   12   and   name   O  )   (resid   16   and   name   HN  )   1.95   0.25   0.25
assign   (resid   12   and   name   O  )   (resid   16   and   name   N   )   2.95   0.25   0.25
assign   (resid   13   and   name   O  )   (resid   17   and   name   HN  )   1.95   0.25   0.25
assign   (resid   13   and   name   O  )   (resid   17   and   name   N   )   2.95   0.25   0.25
assign   (resid   14   and   name   O  )   (resid   18   and   name   HN  )   1.95   0.25   0.25
assign   (resid   14   and   name   O  )   (resid   18   and   name   N   )   2.95   0.25   0.25
assign   (resid   29   and   name   O  )   (resid   33   and   name   HN  )   1.95   0.25   0.25
assign   (resid   29   and   name   O  )   (resid   33   and   name   N   )   2.95   0.25   0.25
assign   (resid   30   and   name   O  )   (resid   34   and   name   HN  )   1.95   0.25   0.25
assign   (resid   30   and   name   O  )   (resid   34   and   name   N   )   2.95   0.25   0.25
assign   (resid   31   and   name   O  )   (resid   35   and   name   HN  )   1.95   0.25   0.25
assign   (resid   31   and   name   O  )   (resid   35   and   name   N   )   2.95   0.25   0.25
assign   (resid   34   and   name   O  )   (resid   37   and   name   HN  )   1.95   0.25   0.25
assign   (resid   34   and   name   O  )   (resid   37   and   name   N   )   2.95   0.25   0.25
assign   (resid   51   and   name   O  )   (resid   55   and   name   HN  )   1.95   0.25   0.25
assign   (resid   51   and   name   O  )   (resid   55   and   name   N   )   2.95   0.25   0.25
assign   (resid   52   and   name   O  )   (resid   56   and   name   HN  )   1.95   0.25   0.25
assign   (resid   52   and   name   O  )   (resid   56   and   name   N   )   2.95   0.25   0.25
assign   (resid   53   and   name   O  )   (resid   57   and   name   HN  )   1.95   0.25   0.25
assign   (resid   53   and   name   O  )   (resid   57   and   name   N   )   2.95   0.25   0.25
assign   (resid   54   and   name   O  )   (resid   58   and   name   HN  )   1.95   0.25   0.25
assign   (resid   54   and   name   O  )   (resid   58   and   name   N   )   2.95   0.25   0.25
assign   (resid   55   and   name   O  )   (resid   59   and   name   HN  )   1.95   0.25   0.25
assign   (resid   55   and   name   O  )   (resid   59   and   name   N   )   2.95   0.25   0.25
assign   (resid   56   and   name   O  )   (resid   60   and   name   HN  )   1.95   0.25   0.25
assign   (resid   56   and   name   O  )   (resid   60   and   name   N   )   2.95   0.25   0.25
assign   (resid   57   and   name   O  )   (resid   61   and   name   HN  )   1.95   0.25   0.25
assign   (resid   57   and   name   O  )   (resid   61   and   name   N   )   2.95   0.25   0.25
assign   (resid   58   and   name   O  )   (resid   62   and   name   HN  )   1.95   0.25   0.25
assign   (resid   58   and   name   O  )   (resid   62   and   name   N   )   2.95   0.25   0.25

set echo = on end
set message = on end
```

!RNA intra- and inter- residue restraints, L30N_noe.tbl ,7/10/98
set echo = off end
set message = off end
!hyrogen bond restraints
!G6-U28

assign	(resid	206	and	name	H1)	(resid	228	and	name	O2)	1.95	0.25	0.25
assign	(resid	206	and	name	N1)	(resid	228	and	name	O2)	2.95	0.25	0.25
assign	(resid	206	and	name	O6)	(resid	228	and	name	H3)	1.95	0.25	0.25
assign	(resid	206	and	name	O6)	(resid	228	and	name	N3)	2.95	0.25	0.25

!G7-G24

assign	(resid	207	and	name	H1)	(resid	224	and	name	N7)	1.95	0.25	0.25
assign	(resid	207	and	name	N1)	(resid	224	and	name	N7)	2.95	0.25	0.25
assign	(resid	207	and	name	H22)	(resid	224	and	name	O6)	1.95	0.25	0.25
assign	(resid	207	and	name	N2)	(resid	224	and	name	O6)	2.95	0.25	0.25

!tetraloop G14-A17

assign	(resid	214	and	name	H21)	(resid	217	and	name	N7)	1.95	0.25	0.25
assign	(resid	214	and	name	N2)	(resid	217	and	name	N7)	2.95	0.25	0.25
assign	(resid	214	and	name	N3)	(resid	217	and	name	H61)	1.95	0.25	0.25
assign	(resid	214	and	name	N3)	(resid	217	and	name	N6)	2.95	0.25	0.25

!tetraloop

assign	(resid	214	and	name	HO2')	(resid	216	and	name	N7)	1.95	0.25	0.25
assign	(resid	214	and	name	O2')	(resid	216	and	name	N7)	2.95	0.25	0.25
assign	(resid	215	and	name	HO2')	(resid	216	and	name	O4')	1.95	0.25	0.25
assign	(resid	215	and	name	O2')	(resid	216	and	name	O4')	2.95	0.25	0.25
assign	(resid	214	and	name	H22)	(resid	217	and	name	O2P)	1.95	0.25	0.25
assign	(resid	214	and	name	N2)	(resid	217	and	name	O2P)	2.95	0.25	0.25

!U10-G21

assign	(resid	210	and	name	H3)	(resid	221	and	name	O6)	1.95	0.25	0.25
assign	(resid	210	and	name	N3)	(resid	221	and	name	O6)	2.95	0.25	0.25
assign	(resid	210	and	name	O2)	(resid	221	and	name	H1)	1.95	0.25	0.25
assign	(resid	210	and	name	O2)	(resid	221	and	name	N1)	2.95	0.25	0.25

!G1-C33

assign	(resid	201	and	name	O6)	(resid	233	and	name	H42)	1.95	0.25	0.25
assign	(resid	201	and	name	O6)	(resid	233	and	name	N4)	2.95	0.25	0.25
assign	(resid	201	and	name	H1)	(resid	233	and	name	N3)	1.95	0.25	0.25
assign	(resid	201	and	name	N1)	(resid	233	and	name	N3)	2.95	0.25	0.25
assign	(resid	201	and	name	H22)	(resid	233	and	name	O2)	1.95	0.25	0.25
assign	(resid	201	and	name	N2)	(resid	233	and	name	O2)	2.95	0.25	0.25

!G2-C32

assign	(resid	202	and	name	O6)	(resid	232	and	name	H42)	1.95	0.25	0.25
assign	(resid	202	and	name	O6)	(resid	232	and	name	N4)	2.95	0.25	0.25
assign	(resid	202	and	name	H1)	(resid	232	and	name	N3)	1.95	0.25	0.25
assign	(resid	202	and	name	N1)	(resid	232	and	name	N3)	2.95	0.25	0.25
assign	(resid	202	and	name	H22)	(resid	232	and	name	O2)	1.95	0.25	0.25
assign	(resid	202	and	name	N2)	(resid	232	and	name	O2)	2.95	0.25	0.25

!A3-U31

assign	(resid	203	and	name	H62)	(resid	231	and	name	O4)	1.95	0.25	0.25
assign	(resid	203	and	name	N6)	(resid	231	and	name	O4)	2.95	0.25	0.25
assign	(resid	203	and	name	N1)	(resid	231	and	name	H3)	1.95	0.25	0.25
assign	(resid	203	and	name	N1)	(resid	231	and	name	N3)	2.95	0.25	0.25
assign	(resid	203	and	name	H2)	(resid	231	and	name	O2)	2.95	0.25	0.25

!C4-G30

assign	(resid	204	and	name	H42)	(resid	230	and	name	O6)	1.95	0.25	0.25
assign	(resid	204	and	name	N4)	(resid	230	and	name	O6)	2.95	0.25	0.25
assign	(resid	204	and	name	N3)	(resid	230	and	name	H1)	1.95	0.25	0.25
assign	(resid	204	and	name	N3)	(resid	230	and	name	N1)	2.95	0.25	0.25
assign	(resid	204	and	name	O2)	(resid	230	and	name	H22)	1.95	0.25	0.25
assign	(resid	204	and	name	O2)	(resid	230	and	name	N2)	2.95	0.25	0.25

!C5-G29

assign	(resid	205	and	name	H42)	(resid	229	and	name	O6)	1.95	0.25	0.25
assign	(resid	205	and	name	N4)	(resid	229	and	name	O6)	2.95	0.25	0.25
assign	(resid	205	and	name	N3)	(resid	229	and	name	H1)	1.95	0.25	0.25
assign	(resid	205	and	name	N3)	(resid	229	and	name	N1)	2.95	0.25	0.25
assign	(resid	205	and	name	O2)	(resid	229	and	name	H22)	1.95	0.25	0.25
assign	(resid	205	and	name	O2)	(resid	229	and	name	N2)	2.95	0.25	0.25

!G9-C22

assign	(resid	209	and	name	O6)	(resid	222	and	name	H42)	1.95	0.25	0.25
assign	(resid	209	and	name	O6)	(resid	222	and	name	N4)	2.95	0.25	0.25
assign	(resid	209	and	name	H1)	(resid	222	and	name	N3)	1.95	0.25	0.25
assign	(resid	209	and	name	N1)	(resid	222	and	name	N3)	2.95	0.25	0.25
assign	(resid	209	and	name	H22)	(resid	222	and	name	O2)	1.95	0.25	0.25
assign	(resid	209	and	name	N2)	(resid	222	and	name	O2)	2.95	0.25	0.25

!G11-C20

assign	(resid	211	and	name	O6)	(resid	220	and	name	H42)	1.95	0.25	0.25
assign	(resid	211	and	name	O6)	(resid	220	and	name	N4)	2.95	0.25	0.25
assign	(resid	211	and	name	H1)	(resid	220	and	name	N3)	1.95	0.25	0.25
assign	(resid	211	and	name	N1)	(resid	220	and	name	N3)	2.95	0.25	0.25

assign	(resid	211	and	name	H22)	(resid	220	and	name	O2)	1.95	0.25	0.25
assign	(resid	211	and	name	N2)	(resid	220	and	name	O2)	2.95	0.25	0.25

!U12-A19

assign	(resid	212	and	name	H3)	(resid	219	and	name	N1)	1.95	0.25	0.25
assign	(resid	212	and	name	N3)	(resid	219	and	name	N1)	2.95	0.25	0.25
assign	(resid	212	and	name	O4)	(resid	219	and	name	H62)	1.95	0.25	0.25
assign	(resid	212	and	name	O4)	(resid	219	and	name	N6)	2.95	0.25	0.25
assign	(resid	212	and	name	O2)	(resid	219	and	name	H2)	2.95	0.25	0.25

!C13-G18

assign	(resid	213	and	name	H42)	(resid	218	and	name	O6)	1.95	0.25	0.25
assign	(resid	213	and	name	N4)	(resid	218	and	name	O6)	2.95	0.25	0.25
assign	(resid	213	and	name	N3)	(resid	218	and	name	H1)	1.95	0.25	0.25
assign	(resid	213	and	name	N3)	(resid	218	and	name	N1)	2.95	0.25	0.25
assign	(resid	213	and	name	O2)	(resid	218	and	name	H22)	1.95	0.25	0.25
assign	(resid	213	and	name	O2)	(resid	218	and	name	N2)	2.95	0.25	0.25

!imino and aminos to others

!G1

assign	(resid	201	and	name	H1)	(resid	202	and	name	H1)	2.9	1.1	1.1
assign	(resid	201	and	name	H1)	(resid	233	and	name	H41)	2.9	1.1	1.1
assign	(resid	201	and	name	H1)	(resid	233	and	name	H42)	2.4	0.6	0.6
assign	(resid	201	and	name	H1)	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	201	and	name	H1)	(resid	232	and	name	H42)	3.4	1.6	1.6
assign	(resid	201	and	name	H21)	(resid	202	and	name	H1')	2.9	1.1	1.1

!G2

assign	(resid	202	and	name	H1)	(resid	231	and	name	H3)	3.4	1.6	1.6
assign	(resid	202	and	name	H1)	(resid	232	and	name	H41)	2.9	1.1	1.1
assign	(resid	202	and	name	H1)	(resid	232	and	name	H42)	2.4	0.6	0.6
assign	(resid	202	and	name	H1)	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	202	and	name	H1)	(resid	203	and	name	H2)	3.4	1.6	1.6
assign	(resid	202	and	name	H21)	(resid	203	and	name	H1')	2.9	1.1	1.1
assign	(resid	202	and	name	H22)	(resid	233	and	name	H1')	2.9	1.1	1.1

!G7

assign	(resid	207	and	name	H1)	(resid	207	and	name	H1')	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	208	and	name	H1')	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	208	and	name	H8)	3.4	1.6	1.6
assign	(resid	207	and	name	H1)	(resid	208	and	name	H2)	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	224	and	name	H8)	2.9	1.1	1.1
assign	(resid	207	and	name	H21)	(resid	207	and	name	H1')	2.9	1.1	1.1

!G9

assign	(resid	209	and	name	H1)	(resid	210	and	name	H3)	2.9	1.1	1.1
assign	(resid	209	and	name	H1)	(resid	221	and	name	H1)	2.9	1.1	1.1
assign	(resid	209	and	name	H1)	(resid	222	and	name	H41)	2.9	1.1	1.1
assign	(resid	209	and	name	H1)	(resid	222	and	name	H42)	2.4	0.6	0.6
assign	(resid	209	and	name	H1)	(resid	222	and	name	H5)	3.4	1.6	1.6
assign	(resid	209	and	name	H1)	(resid	223	and	name	H2)	2.9	1.1	1.1
assign	(resid	209	and	name	H21)	(resid	209	and	name	H2')	3.4	1.6	1.6
assign	(resid	209	and	name	H21)	(resid	210	and	name	H1')	3.4	1.6	1.6
assign	(resid	209	and	name	H21)	(resid	223	and	name	H1')	2.9	1.1	1.1
assign	(resid	209	and	name	H21)	(resid	227	and	name	H1')	2.9	1.1	1.1
assign	(resid	209	and	name	H22)	(resid	222	and	name	H2')	2.9	1.1	1.1

!U10

assign	(resid	210	and	name	H3)	(resid	211	and	name	H1)	3.4	1.6	1.6
assign	(resid	210	and	name	H3)	(resid	211	and	name	H8)	3.4	1.6	1.6
assign	(resid	210	and	name	H3)	(resid	211	and	name	H1')	3.9	2.1	2.1
assign	(resid	210	and	name	H3)	(resid	221	and	name	H1)	2.4	0.6	0.6
assign	(resid	210	and	name	H3)	(resid	221	and	name	H22)	2.9	1.1	1.1
assign	(resid	210	and	name	H3)	(resid	222	and	name	H1')	3.9	2.1	2.1

!G11

assign	(resid	211	and	name	H1)	(resid	211	and	name	H2')	3.9	2.1	2.1
assign	(resid	211	and	name	H1)	(resid	212	and	name	H3)	2.9	1.1	1.1
assign	(resid	211	and	name	H1)	(resid	212	and	name	H1')	3.9	2.1	2.1
assign	(resid	211	and	name	H1)	(resid	219	and	name	H2)	3.4	1.6	1.6
assign	(resid	211	and	name	H1)	(resid	220	and	name	H41)	2.9	1.1	1.1
assign	(resid	211	and	name	H1)	(resid	220	and	name	H42)	2.4	0.6	0.6
assign	(resid	211	and	name	H1)	(resid	220	and	name	H5)	3.9	2.1	2.1
assign	(resid	211	and	name	H1)	(resid	221	and	name	H1)	3.4	1.6	1.6
assign	(resid	211	and	name	H1)	(resid	221	and	name	H8)	3.4	1.6	1.6
assign	(resid	211	and	name	H1)	(resid	221	and	name	H2')	3.9	2.1	2.1
assign	(resid	211	and	name	H21)	(resid	221	and	name	H1')	3.4	1.6	1.6
assign	(resid	211	and	name	H21)	(resid	212	and	name	H1')	3.4	1.6	1.6

!U12

assign	(resid	212	and	name	H3)	(resid	212	and	name	H1')	3.4	1.6	1.6
assign	(resid	212	and	name	H3)	(resid	213	and	name	H5)	3.9	2.1	2.1
assign	(resid	212	and	name	H3)	(resid	213	and	name	H42)	2.9	1.1	1.1
assign	(resid	212	and	name	H3)	(resid	213	and	name	H41)	3.4	1.6	1.6
assign	(resid	212	and	name	H3)	(resid	218	and	name	H1)	3.4	1.6	1.6
assign	(resid	212	and	name	H3)	(resid	219	and	name	H2)	2.4	0.6	0.6

assign	(resid	212	and	name	H3)	(resid	220	and	name	H42)	2.9	1.1	1.1
assign	(resid	212	and	name	H3)	(resid	220	and	name	H41)	3.4	1.6	1.6

!G14

assign	(resid	214	and	name	H1)	(resid	217	and	name	H8)	3.4	1.6	1.6
assign	(resid	214	and	name	H1)	(resid	216	and	name	H8)	3.9	2.1	2.1

!G18

assign	(resid	218	and	name	H1)	(resid	213	and	name	H41)	2.9	1.1	1.1
assign	(resid	218	and	name	H1)	(resid	213	and	name	H42)	2.4	0.6	0.6
assign	(resid	218	and	name	H1)	(resid	213	and	name	H5)	3.4	1.6	1.6
assign	(resid	218	and	name	H1)	(resid	219	and	name	H2)	3.4	1.6	1.6
assign	(resid	218	and	name	H21)	(resid	218	and	name	H2')	3.4	1.6	1.6
assign	(resid	218	and	name	H21)	(resid	219	and	name	H1')	2.9	1.1	1.1
assign	(resid	218	and	name	H21)	(resid	214	and	name	H1')	2.9	1.1	1.1

!G21

assign	(resid	221	and	name	H1)	(resid	211	and	name	H8)	2.9	1.1	1.1
assign	(resid	221	and	name	H1)	(resid	221	and	name	H2*)	2.4	0.6	0.6
assign	(resid	221	and	name	H21)	(resid	211	and	name	H1')	2.4	0.6	0.6
assign	(resid	221	and	name	H21)	(resid	222	and	name	H1')	2.9	1.1	1.1

!G24

assign	(resid	224	and	name	H1)	(resid	223	and	name	H2)	3.4	1.6	1.6
assign	(resid	224	and	name	H1)	(resid	208	and	name	H2)	2.9	1.1	1.1
assign	(resid	224	and	name	H21)	(resid	223	and	name	H2')	2.9	1.1	1.1

!G29

assign	(resid	229	and	name	H1)	(resid	205	and	name	H41)	2.9	1.1	1.1
assign	(resid	229	and	name	H1)	(resid	205	and	name	H42)	2.4	0.6	0.6
assign	(resid	229	and	name	H1)	(resid	205	and	name	H5)	3.4	1.6	1.6
assign	(resid	229	and	name	H1)	(resid	229	and	name	H2')	3.9	2.1	2.1
assign	(resid	229	and	name	H1)	(resid	206	and	name	H2')	3.4	1.6	1.6
assign	(resid	229	and	name	H21)	(resid	206	and	name	H1')	2.9	1.1	1.1
assign	(resid	229	and	name	H21)	(resid	230	and	name	H1')	2.9	1.1	1.1
assign	(resid	229	and	name	H1)	(resid	230	and	name	H1)	2.9	1.1	1.1

!G30

assign	(resid	230	and	name	H1)	(resid	230	and	name	H2')	3.9	2.1	2.1
assign	(resid	230	and	name	H1)	(resid	204	and	name	H41)	2.9	1.1	1.1
assign	(resid	230	and	name	H1)	(resid	204	and	name	H42)	2.4	0.6	0.6
assign	(resid	230	and	name	H1)	(resid	205	and	name	H41)	3.4	1.6	1.6
assign	(resid	230	and	name	H1)	(resid	205	and	name	H42)	3.4	1.6	1.6
assign	(resid	230	and	name	H1)	(resid	204	and	name	H5)	3.4	1.6	1.6
assign	(resid	230	and	name	H1)	(resid	203	and	name	H2)	3.4	1.6	1.6
assign	(resid	230	and	name	H1)	(resid	204	and	name	H2')	3.4	1.6	1.6
assign	(resid	230	and	name	H21)	(resid	205	and	name	H1')	2.9	1.1	1.1
assign	(resid	230	and	name	H21)	(resid	231	and	name	H1')	2.9	1.1	1.1
assign	(resid	230	and	name	H21)	(resid	230	and	name	H2')	3.4	1.6	1.6
assign	(resid	230	and	name	H22)	(resid	204	and	name	H2')	3.4	1.6	1.6
assign	(resid	230	and	name	H1)	(resid	.231	and	name	H3)	2.9	1.1	1.1

!U31

assign	(resid	231	and	name	H3)	(resid	231	and	name	H1')	3.4	1.6	1.6
assign	(resid	231	and	name	H3)	(resid	231	and	name	H2')	3.4	1.6	1.6
assign	(resid	231	and	name	H3)	(resid	203	and	name	H2)	2.4	0.6	0.6
assign	(resid	231	and	name	H3)	(resid	204	and	name	H5)	3.9	2.1	2.1
assign	(resid	231	and	name	H3)	(resid	204	and	name	H42)	2.9	1.1	1.1
assign	(resid	231	and	name	H3)	(resid	204	and	name	H41)	3.9	2.1	2.1
assign	(resid	231	and	name	H3)	(resid	232	and	name	H42)	2.9	1.1	1.1
assign	(resid	231	and	name	H3)	(resid	232	and	name	H41)	2.9	1.1	1.1

!C4

assign	(resid	204	and	name	H41)	(resid	204	and	name	H5)	2.4	0.6	0.6

!C5

assign	(resid	205	and	name	H41)	(resid	205	and	name	H5)	2.4	0.6	0.6

!C13

assign	(resid	213	and	name	H41)	(resid	213	and	name	H5)	2.4	0.6	0.6

!C20

assign	(resid	220	and	name	H41)	(resid	220	and	name	H5)	2.4	0.6	0.6

!C22

assign	(resid	222	and	name	H41)	(resid	222	and	name	H5)	2.4	0.6	0.6

!C32

assign	(resid	232	and	name	H41)	(resid	232	and	name	H5)	2.4	0.6	0.6

!C33

assign	(resid	233	and	name	H41)	(resid	233	and	name	H5)	2.4	0.6	0.6

!non-exchangeable restraints
!first set include intra-nucleotide restraints
!second set include inter-nucleotide restraints
!G1

assign	(resid	201	and	name	H1')	(resid	201	and	name	H2')	2.4	0.6	0.6
assign	(resid	201	and	name	H1')	(resid	201	and	name	H3')	3.4	1.6	1.6
assign	(resid	201	and	name	H1')	(resid	201	and	name	H4')	2.9	1.1	1.1
assign	(resid	201	and	name	H1')	(resid	201	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	201	and	name	H1')	(resid	201	and	name	H8)	3.4	1.6	1.6

assign	(resid	201	and	name	H2')	(resid	201	and	name	H3')	2.9	1.1	1.1
assign	(resid	201	and	name	H2')	(resid	201	and	name	H4')	3.4	1.6	1.6
assign	(resid	201	and	name	H2')	(resid	201	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	201	and	name	H2')	(resid	201	and	name	H8)	2.9	1.1	1.1
assign	(resid	201	and	name	H3')	(resid	201	and	name	H4')	2.4	0.6	0.6
assign	(resid	201	and	name	H3')	(resid	201	and	name	H8)	3.4	1.6	1.6
assign	(resid	201	and	name	H4')	(resid	201	and	name	H8)	3.9	2.1	2.1
assign	(resid	201	and	name	H4')	(resid	201	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	201	and	name	H5'*)	(resid	201	and	name	H8)	2.9	1.1	1.1
assign	(resid	201	and	name	H1')	(resid	202	and	name	H1')	3.9	2.1	2.1
assign	(resid	201	and	name	H1')	(resid	202	and	name	H8)	2.9	1.1	1.1
assign	(resid	201	and	name	H2')	(resid	202	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	201	and	name	H2')	(resid	202	and	name	H8)	2.4	0.6	0.6
assign	(resid	201	and	name	H3')	(resid	202	and	name	H8)	3.4	1.6	1.6
assign	(resid	201	and	name	H8)	(resid	202	and	name	H8)	3.4	1.6	1.6

!G2

assign	(resid	202	and	name	H1')	(resid	202	and	name	H2')	2.4	0.6	0.6
assign	(resid	202	and	name	H1')	(resid	202	and	name	H3')	2.9	1.1	1.1
assign	(resid	202	and	name	H1')	(resid	202	and	name	H4')	2.9	1.1	1.1
assign	(resid	202	and	name	H1')	(resid	202	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	202	and	name	H1')	(resid	202	and	name	H8)	2.9	1.1	1.1
assign	(resid	202	and	name	H2')	(resid	202	and	name	H3')	2.9	1.1	1.1
assign	(resid	202	and	name	H2')	(resid	202	and	name	H4')	2.9	1.1	1.1
assign	(resid	202	and	name	H2')	(resid	202	and	name	H8)	3.4	1.6	1.6
assign	(resid	202	and	name	H3')	(resid	202	and	name	H8)	2.9	1.1	1.1
assign	(resid	202	and	name	H4')	(resid	202	and	name	H8)	3.4	1.6	1.6
assign	(resid	202	and	name	H5'*)	(resid	202	and	name	H8)	2.9	1.1	1.1
assign	(resid	202	and	name	H1')	(resid	203	and	name	H1')	3.9	2.1	2.1
assign	(resid	202	and	name	H1')	(resid	203	and	name	H8)	3.4	1.6	1.6
assign	(resid	202	and	name	H2')	(resid	203	and	name	H8)	2.4	0.6	0.6
assign	(resid	202	and	name	H3')	(resid	203	and	name	H8)	3.9	2.1	2.1
assign	(resid	202	and	name	H4')	(resid	203	and	name	H8)	3.9	2.1	2.1
assign	(resid	202	and	name	H5'*)	(resid	201	and	name	H2')	3.4	1.6	1.6
assign	(resid	202	and	name	H8)	(resid	203	and	name	H8)	3.9	2.1	2.1

!A3

assign	(resid	203	and	name	H1')	(resid	203	and	name	H2')	2.4	0.6	0.6
assign	(resid	203	and	name	H1')	(resid	203	and	name	H3')	2.9	1.1	1.1
assign	(resid	203	and	name	H1')	(resid	203	and	name	H4')	2.9	1.1	1.1
assign	(resid	203	and	name	H1')	(resid	203	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	203	and	name	H1')	(resid	203	and	name	H8)	2.9	1.1	1.1
assign	(resid	203	and	name	H2')	(resid	203	and	name	H3')	2.9	1.1	1.1
assign	(resid	203	and	name	H2')	(resid	203	and	name	H4')	2.9	1.1	1.1
assign	(resid	203	and	name	H2')	(resid	203	and	name	H8)	3.4	1.6	1.6
assign	(resid	203	and	name	H3')	(resid	203	and	name	H8)	2.9	1.1	1.1
assign	(resid	203	and	name	H4')	(resid	203	and	name	H8)	3.4	1.6	1.6
assign	(resid	203	and	name	H5'*)	(resid	203	and	name	H8)	2.9	1.1	1.1
assign	(resid	203	and	name	H1')	(resid	204	and	name	H1')	3.9	2.1	2.1
assign	(resid	203	and	name	H1')	(resid	204	and	name	H5)	3.9	2.1	2.1
assign	(resid	203	and	name	H1')	(resid	204	and	name	H6)	3.9	2.1	2.1
assign	(resid	203	and	name	H2')	(resid	204	and	name	H1')	3.9	2.1	2.1
assign	(resid	203	and	name	H2')	(resid	204	and	name	H5)	2.9	1.1	1.1
assign	(resid	203	and	name	H2')	(resid	204	and	name	H6)	2.4	0.6	0.6
assign	(resid	203	and	name	H3')	(resid	204	and	name	H5)	3.9	2.1	2.1
assign	(resid	203	and	name	H3')	(resid	204	and	name	H6)	3.4	1.6	1.6
assign	(resid	203	and	name	H2)	(resid	204	and	name	H1')	2.9	1.1	1.1
assign	(resid	203	and	name	H2)	(resid	232	and	name	H1')	2.9	1.1	1.1
assign	(resid	203	and	name	H8)	(resid	204	and	name	H8)	3.4	1.6	1.6

!C4

assign	(resid	204	and	name	H1')	(resid	204	and	name	H2')	2.4	0.6	0.6
assign	(resid	204	and	name	H1')	(resid	204	and	name	H3')	2.9	1.1	1.1
assign	(resid	204	and	name	H1')	(resid	204	and	name	H4')	2.9	1.1	1.1
assign	(resid	204	and	name	H1')	(resid	204	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	204	and	name	H1')	(resid	204	and	name	H6)	2.9	1.1	1.1
assign	(resid	204	and	name	H2')	(resid	204	and	name	H3')	2.9	1.1	1.1
assign	(resid	204	and	name	H2')	(resid	204	and	name	H4')	3.4	1.6	1.6
assign	(resid	204	and	name	H2')	(resid	204	and	name	H6)	2.9	1.1	1.1
assign	(resid	204	and	name	H3')	(resid	204	and	name	H4')	2.4	0.6	0.6
assign	(resid	204	and	name	H3')	(resid	204	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	204	and	name	H3')	(resid	204	and	name	H6)	2.4	0.6	0.6
assign	(resid	204	and	name	H4')	(resid	204	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	204	and	name	H4')	(resid	204	and	name	H6)	3.4	1.6	1.6
assign	(resid	204	and	name	H5'*)	(resid	204	and	name	H6)	2.9	1.1	1.1
assign	(resid	204	and	name	H5'*)	(resid	204	and	name	H5)	3.9	2.1	2.1
assign	(resid	204	and	name	H5)	(resid	204	and	name	H6)	2.4	0.6	0.6

assign	(resid	204	and	name	H1')	(resid	205	and	name	H1')	3.9	2.1	2.1
assign	(resid	204	and	name	H1')	(resid	205	and	name	H5)	3.9	2.1	2.1
assign	(resid	204	and	name	H1')	(resid	205	and	name	H6)	3.4	1.6	1.6
assign	(resid	204	and	name	H2')	(resid	205	and	name	H1')	3.4	1.6	1.6
assign	(resid	204	and	name	H2')	(resid	205	and	name	H5)	3.4	1.6	1.6
assign	(resid	204	and	name	H2')	(resid	205	and	name	H6)	2.4	0.6	0.6
assign	(resid	204	and	name	H3')	(resid	205	and	name	H6)	3.4	1.6	1.6
assign	(resid	204	and	name	H6)	(resid	205	and	name	H5)	3.4	1.6	1.6
assign	(resid	204	and	name	H6)	(resid	205	and	name	H6)	3.4	1.6	1.6
!C5													
assign	(resid	205	and	name	H1')	(resid	205	and	name	H2')	2.4	0.6	0.6
assign	(resid	205	and	name	H1')	(resid	205	and	name	H3')	2.9	1.1	1.1
assign	(resid	205	and	name	H1')	(resid	205	and	name	H4')	2.9	1.1	1.1
assign	(resid	205	and	name	H1')	(resid	205	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	205	and	name	H1')	(resid	205	and	name	H6)	2.9	1.1	1.1
assign	(resid	205	and	name	H2')	(resid	205	and	name	H3')	2.9	1.1	1.1
assign	(resid	205	and	name	H2')	(resid	205	and	name	H6)	3.4	1.6	1.6
assign	(resid	205	and	name	H3')	(resid	205	and	name	H4')	2.9	1.1	1.1
assign	(resid	205	and	name	H3')	(resid	205	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	205	and	name	H3')	(resid	205	and	name	H6)	2.4	0.6	0.6
assign	(resid	205	and	name	H4')	(resid	205	and	name	H6)	3.9	2.1	2.1
assign	(resid	205	and	name	H5'*)	(resid	205	and	name	H6)	2.9	1.1	1.1
assign	(resid	205	and	name	H6)	(resid	205	and	name	H5)	2.4	0.6	0.6
assign	(resid	205	and	name	H1')	(resid	206	and	name	H1')	3.9	2.1	2.1
assign	(resid	205	and	name	H1')	(resid	206	and	name	H8)	2.9	1.1	1.1
assign	(resid	205	and	name	H2')	(resid	206	and	name	H8)	2.9	1.1	1.1
assign	(resid	205	and	name	H3')	(resid	206	and	name	H8)	3.4	1.6	1.6
assign	(resid	205	and	name	H6)	(resid	206	and	name	H8)	3.4	1.6	1.6
!G6													
assign	(resid	206	and	name	H1')	(resid	206	and	name	H2')	2.4	0.6	0.6
assign	(resid	206	and	name	H1')	(resid	206	and	name	H3')	3.4	1.6	1.6
assign	(resid	206	and	name	H1')	(resid	206	and	name	H4')	2.9	1.1	1.1
assign	(resid	206	and	name	H1')	(resid	206	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	206	and	name	H1')	(resid	206	and	name	H8)	3.4	1.6	1.6
assign	(resid	206	and	name	H2')	(resid	206	and	name	H3')	2.4	0.6	0.6
assign	(resid	206	and	name	H2')	(resid	206	and	name	H4')	3.4	1.6	1.6
assign	(resid	206	and	name	H2')	(resid	206	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	206	and	name	H2')	(resid	206	and	name	H8)	3.9	2.1	2.1
assign	(resid	206	and	name	H3')	(resid	206	and	name	H4')	2.9	1.1	1.1
assign	(resid	206	and	name	H3')	(resid	206	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	206	and	name	H3')	(resid	206	and	name	H8)	2.9	1.1	1.1
assign	(resid	206	and	name	H4')	(resid	206	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	206	and	name	H4')	(resid	206	and	name	H8)	3.9	2.1	2.1
assign	(resid	206	and	name	H5'*)	(resid	206	and	name	H8)	3.4	1.6	1.6
assign	(resid	206	and	name	H1')	(resid	207	and	name	H8)	3.9	2.1	2.1
assign	(resid	206	and	name	H2')	(resid	207	and	name	H2')	3.9	2.1	2.1
assign	(resid	206	and	name	H2')	(resid	207	and	name	H3')	3.4	1.6	1.6
assign	(resid	206	and	name	H2')	(resid	207	and	name	H8)	3.4	1.6	1.6
assign	(resid	206	and	name	H3')	(resid	207	and	name	H1')	3.9	2.1	2.1
assign	(resid	206	and	name	H3')	(resid	207	and	name	H2')	3.4	1.6	1.6
assign	(resid	206	and	name	H3')	(resid	207	and	name	H3')	3.4	1.6	1.6
assign	(resid	206	and	name	H3')	(resid	207	and	name	H8)	3.4	1.6	1.6
assign	(resid	206	and	name	H8)	(resid	207	and	name	H8)	3.9	2.1	2.1
!G7													
assign	(resid	207	and	name	H1')	(resid	207	and	name	H2')	2.4	0.6	0.6
assign	(resid	207	and	name	H1')	(resid	207	and	name	H3')	3.4	1.6	1.6
assign	(resid	207	and	name	H1')	(resid	207	and	name	H4')	2.9	1.1	1.1
assign	(resid	207	and	name	H1')	(resid	207	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	207	and	name	H1')	(resid	207	and	name	H8)	2.9	1.1	1.1
assign	(resid	207	and	name	H2')	(resid	207	and	name	H3')	3.4	1.6	1.6
assign	(resid	207	and	name	H2')	(resid	207	and	name	H4')	3.9	2.1	2.1
assign	(resid	207	and	name	H2')	(resid	207	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	207	and	name	H2')	(resid	207	and	name	H8)	2.4	0.6	0.6
assign	(resid	207	and	name	H3')	(resid	207	and	name	H4')	2.4	0.6	0.6
assign	(resid	207	and	name	H3')	(resid	207	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	207	and	name	H3')	(resid	207	and	name	H8)	2.9	1.1	1.1
assign	(resid	207	and	name	H4')	(resid	207	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	207	and	name	H4')	(resid	207	and	name	H8)	3.9	2.1	2.1
assign	(resid	207	and	name	H1')	(resid	208	and	name	H8)	2.9	1.1	1.1
assign	(resid	207	and	name	H4')	(resid	208	and	name	H8)	3.4	1.6	1.6
assign	(resid	207	and	name	H8)	(resid	208	and	name	H8)	3.9	2.1	2.1
!A8													
assign	(resid	208	and	name	H1')	(resid	208	and	name	H2')	3.4	1.6	1.6

assign	(resid	208	and	name	H1')	(resid	208	and	name	H3')	3.4	1.6	1.6
assign	(resid	208	and	name	H1')	(resid	208	and	name	H8)	3.9	2.1	2.1
assign	(resid	208	and	name	H2')	(resid	208	and	name	H3')	2.9	1.1	1.1
assign	(resid	208	and	name	H2')	(resid	208	and	name	H4')	2.9	1.1	1.1
assign	(resid	208	and	name	H2')	(resid	208	and	name	H8)	3.9	2.1	2.1
assign	(resid	208	and	name	H3')	(resid	208	and	name	H4')	2.9	1.1	1.1
assign	(resid	208	and	name	H3')	(resid	208	and	name	H8)	3.4	1.6	1.6
assign	(resid	208	and	name	H1')	(resid	209	and	name	H1')	2.9	1.1	1.1
assign	(resid	208	and	name	H1')	(resid	209	and	name	H8)	3.4	1.6	1.6
assign	(resid	208	and	name	H1')	(resid	227	and	name	H2)	3.4	1.6	1.6
assign	(resid	208	and	name	H2)	(resid	223	and	name	H2)	2.9	1.1	1.1
assign	(resid	208	and	name	H2)	(resid	209	and	name	H1')	2.9	1.1	1.1
assign	(resid	208	and	name	H2)	(resid	223	and	name	H1')	2.9	1.1	1.1
assign	(resid	208	and	name	H2)	(resid	227	and	name	H1')	3.4	1.6	1.6
assign	(resid	208	and	name	H8)	(resid	209	and	name	H8)	3.9	2.1	2.1

!G9

assign	(resid	209	and	name	H1')	(resid	209	and	name	H2')	2.9	1.1	1.1
assign	(resid	209	and	name	H1')	(resid	209	and	name	H3')	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	209	and	name	H4')	2.9	1.1	1.1
assign	(resid	209	and	name	H1')	(resid	209	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	209	and	name	H1')	(resid	209 ·	and	name	H8)	2.9	1.1	1.1
assign	(resid	209	and	name	H2')	(resid	209	and	name	H8)	2.9	1.1	1.1
assign	(resid	209	and	name	H3')	(resid	209	and	name	H8)	2.9	1.1	1.1
assign	(resid	209	and	name	H4')	(resid	209	and	name	H2')	3.9	2.1	2.1
assign	(resid	209	and	name	H4')	(resid	209	and	name	H3')	3.9	2.1	2.1
assign	(resid	209	and	name	H4')	(resid	209	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	209	and	name	H4')	(resid	209	and	name	H8)	3.9	2.1	2.1
assign	(resid	209	and	name	H5'*)	(resid	209	and	name	H8)	2.9	1.1	1.1
assign	(resid	209	and	name	H1')	(resid	210	and	name	H1')	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	210	and	name	H5)	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	210	and	name	H6)	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	227	and	name	H1')	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	227	and	name	H2)	3.4	1.6	1.6
assign	(resid	209	and	name	H1')	(resid	223	and	name	H2)	3.4	1.6	1.6
assign	(resid	209	and	name	H2')	(resid	210	and	name	H5)	2.9	1.1	1.1
assign	(resid	209	and	name	H2')	(resid	210	and	name	H6)	2.4	0.6	0.6
assign	(resid	209	and	name	H3')	(resid	210	and	name	H5)	3.4	1.6	1.6
assign	(resid	209	and	name	H3')	(resid	210	and	name	H6)	2.9	1.1	1.1
assign	(resid	209	and	name	H8)	(resid	210	and	name	H6)	3.4	1.6	1.6
assign	(resid	209	and	name	H8)	(resid	210	and	name	H5)	3.4	1.6	1.6
assign	(resid	209	and	name	H8)	(resid	223	and	name	H2)	3.4	1.6	1.6

!U10

assign	(resid	210	and	name	H1')	(resid	·210	and	name	H2')	2.4	0.6	0.6
assign	(resid	210	and	name	H1')	(resid	210	and	name	H3')	2.9	1.1	1.1
assign	(resid	210	and	name	H1')	(resid	210	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	210	and	name	H1')	(resid	210	and	name	H5)	3.4	1.6	1.6
assign	(resid	210	and	name	H1')	(resid	210	and	name	H6)	2.9	1.1	1.1
assign	(resid	210	and	name	H2')	(resid	210	and	name	H5)	3.4	1.6	1.6
assign	(resid	210	and	name	H2')	(resid	210	and	name	H6)	3.4	1.6	1.6
assign	(resid	210	and	name	H3')	(resid	210	and	name	H6)	2.9	1.1	1.1
assign	(resid	210	and	name	H5'*)	(resid	210	and	name	H6)	2.9	1.1	1.1
assign	(resid	210	and	name	H5)	(resid	210	and	name	H2')	3.4	1.6	1.6
assign	(resid	210	and	name	H6)	(resid	210	and	name	H5)	2.4	0.6	0.6
assign	(resid	210	and	name	H1')	(resid	211	and	name	H1')	3.9	2.1	2.1
assign	(resid	210	and	name	H1')	(resid	211	and	name	H8)	3.4	1.6	1.6
assign	(resid	210	and	name	H2')	(resid	211	and	name	H1')	3.4	1.6	1.6
assign	(resid	210	and	name	H2')	(resid	211	and	name	H8)	3.4	1.6	1.6
assign	(resid	210	and	name	H6)	(resid	211	and	name	H8)	3.4	1.6	1.6

!G11

assign	(resid	211	and	name	H1')	(resid	211	and	name	H2')	2.4	0.6	0.6
assign	(resid	211	and	name	H1')	(resid	211	and	name	H3')	2.9	1.1	1.1
assign	(resid	211	and	name	H1')	(resid	211	and	name	H4')	2.4	0.6	0.6
assign	(resid	211	and	name	H1')	(resid	211	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	211	and	name	H1')	(resid	211	and	name	H8)	2.9	1.1	1.1
assign	(resid	211	and	name	H2')	(resid	211	and	name	H8)	3.4	1.6	1.6
assign	(resid	211	and	name	H3')	(resid	211	and	name	H8)	2.4	0.6	0.6
assign	(resid	211	and	name	H4')	(resid	211	and	name	H8)	3.4	1.6	1.6
assign	(resid	211	and	name	H4')	(resid	211	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	211	and	name	H5'*)	(resid	211	and	name	H8)	2.9	1.1	1.1
assign	(resid	211	and	name	H1')	(resid	212	and	name	H1')	3.4	1.6	1.6
assign	(resid	211	and	name	H1')	(resid	212	and	name	H5)	3.4	1.6	1.6
assign	(resid	211	and	name	H1')	(resid	212	and	name	H6)	3.4	1.6	1.6
assign	(resid	211	and	name	H2')	(resid	212	and	name	H5)	3.4	1.6	1.6

assign	(resid	211	and	name	H2')	(resid	212	and	name	H6)	2.4	0.6	0.6
assign	(resid	211	and	name	H3')	(resid	212	and	name	H5)	3.4	1.6	1.6
assign	(resid	211	and	name	H3')	(resid	212	and	name	H6)	2.9	1.1	1.1
assign	(resid	211	and	name	H8)	(resid	212	and	name	H6)	3.9	2.1	2.1
assign	(resid	211	and	name	H8)	(resid	212	and	name	H5)	3.9	2.1	2.1
!U12													
assign	(resid	212	and	name	H1')	(resid	212	and	name	H2')	2.4	0.6	0.6
assign	(resid	212	and	name	H1')	(resid	212	and	name	H3')	3.4	1.6	1.6
assign	(resid	212	and	name	H1')	(resid	212	and	name	H4')	2.9	1.1	1.1
assign	(resid	212	and	name	H1')	(resid	212	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	212	and	name	H1')	(resid	212	and	name	H6)	2.9	1.1	1.1
assign	(resid	212	and	name	H2')	(resid	212	and	name	H3')	2.4	0.6	0.6
assign	(resid	212	and	name	H2')	(resid	212	and	name	H5)	3.9	2.1	2.1
assign	(resid	212	and	name	H2')	(resid	212	and	name	H6)	3.4	1.6	1.6
assign	(resid	212	and	name	H3')	(resid	212	and	name	H6)	2.4	0.6	0.6
assign	(resid	212	and	name	H4')	(resid	212	and	name	H6)	3.4	1.6	1.6
assign	(resid	212	and	name	H5'*)	(resid	212	and	name	H6)	2.9	1.1	1.1
assign	(resid	212	and	name	H6)	(resid	212	and	name	H5)	2.4	0.6	0.6
assign	(resid	212	and	name	H1')	(resid	213	and	name	H1')	3.9	2.1	2.1
assign	(resid	212	and	name	H1')	(resid	213	and	name	H5)	3.4	1.6	1.6
assign	(resid	212	and	name	H1')	(resid	213	and	name	H6)	3.4	1.6	1.6
assign	(resid	212	and	name	H2')	(resid	213	and	name	H1')	3.4	1.6	1.6
assign	(resid	212	and	name	H2')	(resid	213	and	name	H6)	2.4	0.6	0.6
assign	(resid	212	and	name	H2')	(resid	213	and	name	H5)	3.4	1.6	1.6
assign	(resid	212	and	name	H3')	(resid	213	and	name	H6)	2.9	1.1	1.1
assign	(resid	212	and	name	H6)	(resid	213	and	name	H6)	3.4	1.6	1.6
assign	(resid	212	and	name	H6)	(resid	213	and	name	H5)	3.9	2.1	2.1
assign	(resid	212	and	name	H5)	(resid	213	and	name	H6)	3.9	2.1	2.1
!C13													
assign	(resid	213	and	name	H1')	(resid	213	and	name	H2')	2.4	0.6	0.6
assign	(resid	213	and	name	H1')	(resid	213	and	name	H3')	2.9	1.1	1.1
assign	(resid	213	and	name	H1')	(resid	213	and	name	H4')	2.9	1.1	1.1
assign	(resid	213	and	name	H1')	(resid	213	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	213	and	name	H1')	(resid	213	and	name	H6)	2.9	1.1	1.1
assign	(resid	213	and	name	H2')	(resid	213	and	name	H6)	3.4	1.6	1.6
assign	(resid	213	and	name	H3')	(resid	213	and	name	H6)	2.9	1.1	1.1
assign	(resid	213	and	name	H3')	(resid	213	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	213	and	name	H4')	(resid	213	and	name	H6)	3.4	1.6	1.6
assign	(resid	213	and	name	H4')	(resid	213	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	213	and	name	H5'*)	(resid	213	and	name	H6)	2.9	1.1	1.1
assign	(resid	213	and	name	H5)	(resid	213	and	name	H6)	2.4	0.6	0.6
assign	(resid	213	and	name	H1')	(resid	214	and	name	H1')	3.9	2.1	2.1
assign	(resid	213	and	name	H1')	(resid	214	and	name	H8)	2.9	1.1	1.1
assign	(resid	213	and	name	H1')	(resid	219	and	name	H2)	3.4	1.6	1.6
assign	(resid	213	and	name	H2')	(resid	214	and	name	H8)	2.4	0.6	0.6
assign	(resid	213	and	name	H3')	(resid	214	and	name	H8)	2.9	1.1	1.1
!G14													
assign	(resid	214	and	name	H1')	(resid	214	and	name	H2')	2.4	0.6	0.6
assign	(resid	214	and	name	H1')	(resid	214	and	name	H3')	2.9	1.1	1.1
assign	(resid	214	and	name	H1')	(resid	214	and	name	H4')	2.4	0.6	0.6
assign	(resid	214	and	name	H1')	(resid	214	and	name	H8)	2.9	1.1	1.1
assign	(resid	214	and	name	H2')	(resid	214	and	name	H3')	2.9	1.1	1.1
assign	(resid	214	and	name	H2')	(resid	214	and	name	H8)	3.4	1.6	1.6
assign	(resid	214	and	name	H3')	(resid	214	and	name	H4')	2.4	0.6	0.6
assign	(resid	214	and	name	H3')	(resid	214	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	214	and	name	H3')	(resid	214	and	name	H8)	2.9	1.1	1.1
assign	(resid	214	and	name	H4')	(resid	214	and	name	H8)	3.4	1.6	1.6
assign	(resid	214	and	name	H5'*)	(resid	214	and	name	H8)	3.4	1.6	1.6
assign	(resid	214	and	name	H1')	(resid	215	and	name	H5)	3.9	2.1	2.1
assign	(resid	214	and	name	H1')	(resid	216	and	name	H8)	3.4	1.6	1.6
assign	(resid	214	and	name	H2')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	214	and	name	H2')	(resid	215	and	name	H5)	3.4	1.6	1.6
!C15													
assign	(resid	215	and	name	H1')	(resid	215	and	name	H2')	2.4	0.6	0.6
assign	(resid	215	and	name	H1')	(resid	215	and	name	H3')	3.4	1.6	1.6
assign	(resid	215	and	name	H1')	(resid	215	and	name	H4')	2.9	1.1	1.1
assign	(resid	215	and	name	H1')	(resid	215	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	215	and	name	H1')	(resid	215	and	name	H6)	3.4	1.6	1.6
assign	(resid	215	and	name	H2')	(resid	215	and	name	H4')	2.9	1.1	1.1
assign	(resid	215	and	name	H2')	(resid	215	and	name	H3')	2.4	0.6	0.6
assign	(resid	215	and	name	H2')	(resid	215	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	215	and	name	H2')	(resid	215	and	name	H6)	2.9	1.1	1.1
assign	(resid	215	and	name	H3')	(resid	215	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	215	and	name	H3')	(resid	215	and	name	H6)	3.4	1.6	1.6

assign	(resid	215	and	name	H4')	(resid	215	and	name	H6)	3.9	2.1	2.1
assign	(resid	215	and	name	H4')	(resid	215	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	215	and	name	H5'*)	(resid	215	and	name	H6)	2.9	1.1	1.1
assign	(resid	215	and	name	H6)	(resid	215	and	name	H5)	2.4	0.6	0.6
assign	(resid	215	and	name	H1')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	215	and	name	H6)	(resid	216	and	name	H8)	2.4	0.6	0.6
assign	(resid	215	and	name	H2')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	215	and	name	H3')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	215	and	name	H4')	(resid	216	and	name	H8)	3.4	1.6	1.6
assign	(resid	215	and	name	H5'*)	(resid	216	and	name	H8)	3.4	1.6	1.6
assign	(resid	215	and	name	H6)	(resid	216	and	name	H8)	2.9	1.1	1.1

!A16

assign	(resid	216	and	name	H1')	(resid	216	and	name	H2')	2.4	0.6	0.6
assign	(resid	216	and	name	H1')	(resid	216	and	name	H3')	3.4	1.6	1.6
assign	(resid	216	and	name	H1')	(resid	216	and	name	H4')	2.9	1.1	1.1
assign	(resid	216	and	name	H1')	(resid	216	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	216	and	name	H1')	(resid	216	and	name	H2)	3.9	2.1	2.1
assign	(resid	216	and	name	H1')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	216	and	name	H2')	(resid	216	and	name	H3')	2.4	0.6	0.6
assign	(resid	216	and	name	H2')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	216	and	name	H3')	(resid	216	and	name	H4')	2.4	0.6	0.6
assign	(resid	216	and	name	H3')	(resid	216	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	216	and	name	H3')	(resid	216	and	name	H8)	2.9	1.1	1.1
assign	(resid	216	and	name	H4')	(resid	216	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	216	and	name	H4')	(resid	216	and	name	H8)	3.4	1.6	1.6
assign	(resid	216	and	name	H5'*)	(resid	216	and	name	H8)	3.9	2.1	2.1
assign	(resid	216	and	name	H1')	(resid	217	and	name	H8)	3.4	1.6	1.6
assign	(resid	216	and	name	H1')	(resid	217	and	name	H1')	3.9	2.1	2.1
assign	(resid	216	and	name	H2')	(resid	217	and	name	H1')	3.4	1.6	1.6
assign	(resid	216	and	name	H2')	(resid	217	and	name	H8)	2.9	1.1	1.1
assign	(resid	216	and	name	H3')	(resid	217	and	name	H8)	2.9	1.1	1.1
assign	(resid	216	and	name	H2)	(resid	217	and	name	H2)	3.4	1.6	1.6
assign	(resid	216	and	name	H8)	(resid	217	and	name	H8)	3.4	1.6	1.6

!A17

assign	(resid	217	and	name	H1')	(resid	217	and	name	H2')	2.4	0.6	0.6
assign	(resid	217	and	name	H1')	(resid	217	and	name	H3')	3.4	1.6	1.6
assign	(resid	217	and	name	H1')	(resid	217	and	name	H4')	2.9	1.1	1.1
assign	(resid	217	and	name	H1')	(resid	217	and	name	H2)	3.9	2.1	2.1
assign	(resid	217	and	name	H1')	(resid	217	and	name	H8)	2.9	1.1	1.1
assign	(resid	217	and	name	H2')	(resid	217	and	name	H3')	2.9	1.1	1.1
assign	(resid	217	and	name	H2')	(resid	217	and	name	H4')	2.9	1.1	1.1
assign	(resid	217	and	name	H2')	(resid	217	and	name	H8)	3.4	1.6	1.6
assign	(resid	217	and	name	H3')	(resid	217	and	name	H4')	2.9	1.1	1.1
assign	(resid	217	and	name	H3')	(resid	217	and	name	H8)	3.4	1.6	1.6
assign	(resid	217	and	name	H4')	(resid	217	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	217	and	name	H4')	(resid	217	and	name	H8)	3.9	2.1	2.1
assign	(resid	217	and	name	H5'*)	(resid	217	and	name	H8)	3.4	1.6	1.6
assign	(resid	217	and	name	H1')	(resid	216	and	name	H2)	3.4	1.6	1.6
assign	(resid	217	and	name	H1')	(resid	218	and	name	H8)	3.9	2.1	2.1
assign	(resid	217	and	name	H2')	(resid	218	and	name	H8)	3.4	1.6	1.6
assign	(resid	217	and	name	H3')	(resid	218	and	name	H8)	3.4	1.6	1.6
assign	(resid	217	and	name	H8)	(resid	218	and	name	H8)	3.4	1.6	1.6

!G18

assign	(resid	218	and	name	H1')	(resid	218	and	name	H2')	2.4	0.6	0.6
assign	(resid	218	and	name	H1')	(resid	218	and	name	H3')	2.9	1.1	1.1
assign	(resid	218	and	name	H1')	(resid	218	and	name	H4')	3.4	1.6	1.6
assign	(resid	218	and	name	H1')	(resid	218	and	name	H8)	3.4	1.6	1.6
assign	(resid	218	and	name	H2')	(resid	218	and	name	H8)	3.4	1.6	1.6
assign	(resid	218	and	name	H3')	(resid	218	and	name	H8)	2.9	1.1	1.1
assign	(resid	218	and	name	H4')	(resid	218	and	name	H8)	3.4	1.6	1.6
assign	(resid	218	and	name	H5'*)	(resid	218	and	name	H8)	2.9	1.1	1.1
assign	(resid	218	and	name	H1')	(resid	219	and	name	H1')	3.9	2.1	2.1
assign	(resid	218	and	name	H1')	(resid	219	and	name	H8)	3.4	1.6	1.6
assign	(resid	218	and	name	H2')	(resid	219	and	name	H8)	2.4	0.6	0.6
assign	(resid	218	and	name	H3')	(resid	219	and	name	H8)	2.9	1.1	1.1

!A19

assign	(resid	219	and	name	H1')	(resid	219	and	name	H2')	2.4	0.6	0.6
assign	(resid	219	and	name	H1')	(resid	219	and	name	H3')	2.9	1.1	1.1
assign	(resid	219	and	name	H1')	(resid	219	and	name	H4')	2.4	0.6	0.6
assign	(resid	219	and	name	H1')	(resid	219	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	219	and	name	H1')	(resid	219	and	name	H8)	2.9	1.1	1.1
assign	(resid	219	and	name	H2')	(resid	219	and	name	H3')	2.4	0.6	0.6
assign	(resid	219	and	name	H2')	(resid	219	and	name	H4')	3.4	1.6	1.6

assign	(resid	219	and	name	H2')	(resid	219	and	name	H8)	3.4	1.6	1.6
assign	(resid	219	and	name	H3')	(resid	219	and	name	H8)	2.9	1.1	1.1
assign	(resid	219	and	name	H3')	(resid	219	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	219	and	name	H4')	(resid	219	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	219	and	name	H4')	(resid	219	and	name	H8)	3.4	1.6	1.6
assign	(resid	219	and	name	H5'*)	(resid	219	and	name	H8)	2.9	1.1	1.1
assign	(resid	219	and	name	H1')	(resid	220	and	name	H1')	3.9	2.1	2.1
assign	(resid	219	and	name	H1')	(resid	220	and	name	H5)	3.4	1.6	1.6
assign	(resid	219	and	name	H1')	(resid	220	and	name	H6)	3.4	1.6	1.6
assign	(resid	219	and	name	H2')	(resid	220	and	name	H6)	2.4	0.6	0.6
assign	(resid	219	and	name	H2')	(resid	220	and	name	H5)	3.4	1.6	1.6
assign	(resid	219	and	name	H3')	(resid	220	and	name	H6)	2.9	1.1	1.1
assign	(resid	219	and	name	H3')	(resid	220	and	name	H5)	3.4	1.6	1.6
assign	(resid	219	and	name	H2)	(resid	220	and	name	H1')	2.9	1.1	1.1
assign	(resid	219	and	name	H8)	(resid	220	and	name	H6)	3.4	1.6	1.6
!C20													
assign	(resid	220	and	name	H1')	(resid	220	and	name	H2')	2.4	0.6	0.6
assign	(resid	220	and	name	H1')	(resid	220	and	name	H3')	2.9	1.1	1.1
assign	(resid	220	and	name	H1')	(resid	220	and	name	H4')	2.9	1.1	1.1
assign	(resid	220	and	name	H1')	(resid	220	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	220	and	name	H1')	(resid	220	and	name	H6)	3.4	1.6	1.6
assign	(resid	220	and	name	H2')	(resid	220	and	name	H3')	2.9	1.1	1.1
assign	(resid	220	and	name	H2')	(resid	220	and	name	H6)	3.4	1.6	1.6
assign	(resid	220	and	name	H2')	(resid	220	and	name	H5)	3.4	1.6	1.6
assign	(resid	220	and	name	H3')	(resid	220	and	name	H6)	2.4	0.6	0.6
assign	(resid	220	and	name	H3')	(resid	220	and	name	H5)	3.9	2.1	2.1
assign	(resid	220	and	name	H4')	(resid	220	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	220	and	name	H4')	(resid	220	and	name	H6)	3.4	1.6	1.6
assign	(resid	220	and	name	H4')	(resid	220	and	name	H5)	3.9	2.1	2.1
assign	(resid	220	and	name	H5'*)	(resid	220	and	name	H6)	2.9	1.1	1.1
assign	(resid	220	and	name	H5'*)	(resid	220	and	name	H5)	3.4	1.6	1.6
assign	(resid	220	and	name	H6)	(resid	220	and	name	H5)	2.4	0.6	0.6
assign	(resid	220	and	name	H1')	(resid	221	and	name	H1')	3.9	2.1	2.1
assign	(resid	220	and	name	H1')	(resid	221	and	name	H8)	3.4	1.6	1.6
assign	(resid	220	and	name	H2')	(resid	221	and	name	H8)	2.4	0.6	0.6
assign	(resid	220	and	name	H2')	(resid	221	and	name	H1')	3.4	1.6	1.6
assign	(resid	220	and	name	H3')	(resid	221	and	name	H8)	2.9	1.1	1.1
assign	(resid	220	and	name	H6)	(resid	221	and	name	H8)	3.9	2.1	2.1
!G21													
assign	(resid	221	and	name	H1')	(resid	221	and	name	H2')	2.4	0.6	0.6
assign	(resid	221	and	name	H1')	(resid	221	and	name	H3')	3.4	1.6	1.6
assign	(resid	221	and	name	H1')	(resid	221	and	name	H4')	2.4	0.6	0.6
assign	(resid	221	and	name	H1')	(resid	221	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	221	and	name	H1')	(resid	221	and	name	H8)	2.9	1.1	1.1
assign	(resid	221	and	name	H2')	(resid	221	and	name	H3')	2.4	0.6	0.6
assign	(resid	221	and	name	H2')	(resid	221	and	name	H8)	3.4	1.6	1.6
assign	(resid	221	and	name	H3')	(resid	221	and	name	H8)	2.4	0.6	0.6
assign	(resid	221	and	name	H4')	(resid	221	and	name	H8)	3.4	1.6	1.6
assign	(resid	221	and	name	H4')	(resid	221	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	221	and	name	H5'*)	(resid	221	and	name	H8)	2.9	1.1	1.1
assign	(resid	221	and	name	H1')	(resid	222	and	name	H1')	3.9	2.1	2.1
assign	(resid	221	and	name	H1')	(resid	222	and	name	H5)	3.4	1.6	1.6
assign	(resid	221	and	name	H1')	(resid	222	and	name	H6)	3.4	1.6	1.6
assign	(resid	221	and	name	H2')	(resid	222	and	name	H1')	3.9	2.1	2.1
assign	(resid	221	and	name	H2')	(resid	222	and	name	H6)	2.4	0.6	0.6
assign	(resid	221	and	name	H2')	(resid	222	and	name	H5)	3.4	1.6	1.6
assign	(resid	221	and	name	H3')	(resid	222	and	name	H6)	2.9	1.1	1.1
assign	(resid	221	and	name	H3')	(resid	222	and	name	H5)	3.4	1.6	1.6
!C22													
assign	(resid	222	and	name	H1')	(resid	222	and	name	H2')	2.4	0.6	0.6
assign	(resid	222	and	name	H1')	(resid	222	and	name	H3')	2.9	1.1	1.1
assign	(resid	222	and	name	H1')	(resid	222	and	name	H4')	2.9	1.1	1.1
assign	(resid	222	and	name	H1')	(resid	222	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	222	and	name	H1')	(resid	222	and	name	H6)	3.4	1.6	1.6
assign	(resid	222	and	name	H1')	(resid	222	and	name	H5)	3.9	2.1	2.1
assign	(resid	222	and	name	H2')	(resid	222	and	name	H6)	3.4	1.6	1.6
assign	(resid	222	and	name	H3')	(resid	222	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	222	and	name	H3')	(resid	222	and	name	H6)	2.4	0.6	0.6
assign	(resid	222	and	name	H3')	(resid	222	and	name	H5)	3.9	2.1	2.1
assign	(resid	222	and	name	H4')	(resid	222	and	name	H6)	2.9	1.1	1.1
assign	(resid	222	and	name	H4')	(resid	222	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	222	and	name	H5'*)	(resid	222	and	name	H6)	2.9	1.1	1.1
assign	(resid	222	and	name	H5'*)	(resid	222	and	name	H5')	3.4	1.6	1.6
assign	(resid	222	and	name	H6)	(resid	222	and	name	H5)	2.4	0.6	0.6

assign	(resid	222	and	name	H1')	(resid	223	and	name	H8)	3.4	1.6	1.6
assign	(resid	222	and	name	H2')	(resid	223	and	name	H8)	2.4	0.6	0.6
assign	(resid	222	and	name	H3')	(resid	223	and	name	H8)	3.4	1.6	1.6
assign	(resid	222	and	name	H6)	(resid	223	and	name	H8)	3.4	1.6	1.6
assign	(resid	222	and	name	H5)	(resid	223	and	name	H8)	3.9	2.1	2.1

!A23

assign	(resid	223	and	name	H1')	(resid	223	and	name	H2')	2.4	0.6	0.6
assign	(resid	223	and	name	H1')	(resid	223	and	name	H3')	2.9	1.1	1.1
assign	(resid	223	and	name	H1')	(resid	223	and	name	H4')	2.9	1.1	1.1
assign	(resid	223	and	name	H1')	(resid	223	and	name	H2)	3.4	1.6	1.6
assign	(resid	223	and	name	H1')	(resid	223	and	name	H8)	3.4	1.6	1.6
assign	(resid	223	and	name	H2')	(resid	223	and	name	H3')	2.9	1.1	1.1
assign	(resid	223	and	name	H2')	(resid	223	and	name	H2)	3.9	2.1	2.1
assign	(resid	223	and	name	H2')	(resid	223	and	name	H8)	2.9	1.1	1.1
assign	(resid	223	and	name	H2')	(resid	223	and	name	H8)	2.9	1.1	1.1
assign	(resid	223	and	name	H3')	(resid	223	and	name	H8)	2.4	0.6	0.6

assign	(resid	223	and	name	H1')	(resid	227	and	name	H1')	2.9	1.1	1.1
assign	(resid	223	and	name	H1')	(resid	227	and	name	H8)	3.9	2.1	2.1
assign	(resid	223	and	name	H2')	(resid	227	and	name	H1')	3.4	1.6	1.6
assign	(resid	223	and	name	H2')	(resid	224	and	name	H2')	2.9	1.1	1.1
assign	(resid	223	and	name	H2)	(resid	227	and	name	H1')	3.4	1.6	1.6

!G24

assign	(resid	224	and	name	H1')	(resid	224	and	name	H2')	3.4	1.6	1.6
assign	(resid	224	and	name	H1')	(resid	224	and	name	H3')	3.4	1.6	1.6
assign	(resid	224	and	name	H1')	(resid	224	and	name	H4')	3.9	2.1	2.1
assign	(resid	224	and	name	H1')	(resid	224	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	224	and	name	H1')	(resid	224	and	name	H8)	2.4	0.6	0.6
assign	(resid	224	and	name	H2')	(resid	224	and	name	H3')	2.9	1.1	1.1
assign	(resid	224	and	name	H2')	(resid	224	and	name	H4')	3.4	1.6	1.6
assign	(resid	224	and	name	H2')	(resid	224	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	224	and	name	H2')	(resid	224	and	name	H8)	3.4	1.6	1.6
assign	(resid	224	and	name	H3')	(resid	224	and	name	H4')	2.9	1.1	1.1
assign	(resid	224	and	name	H3')	(resid	224	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	224	and	name	H3')	(resid	224	and	name	H8)	3.9	2.1	2.1
assign	(resid	224	and	name	H4')	(resid	224	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	224	and	name	H4')	(resid	224	and	name	H8)	3.9	2.1	2.1

assign	(resid	224	and	name	H2')	(resid	225	and	name	H8)	3.9	2.1	2.1
assign	(resid	224	and	name	H3')	(resid	225	and	name	H1')	3.9	2.1	2.1
assign	(resid	224	and	name	H3')	(resid	225	and	name	H8)	3.4	1.6	1.6
assign	(resid	224	and	name	H4')	(resid	225	and	name	H2')	3.4	1.6	1.6
assign	(resid	224	and	name	H4')	(resid	225	and	name	H3')	2.9	1.1	1.1
assign	(resid	224	and	name	H4')	(resid	225	and	name	H4')	2.9	1.1	1.1
assign	(resid	224	and	name	H4')	(resid	225	and	name	H8)	3.9	2.1	2.1
assign	(resid	224	and	name	H4')	(resid	226	and	name	H1')	3.9	2.1	2.1
assign	(resid	224	and	name	H4')	(resid	226	and	name	H2')	3.4	1.6	1.6
assign	(resid	224	and	name	H4')	(resid	226	and	name	H3')	3.4	1.6	1.6
assign	(resid	224	and	name	H4')	(resid	226	and	name	H4')	3.9	2.1	2.1
assign	(resid	224	and	name	H4')	(resid	226	and	name	H8)	3.4	1.6	1.6

!A25

assign	(resid	225	and	name	H1')	(resid	225	and	name	H2')	2.4	0.6	0.6
assign	(resid	225	and	name	H1')	(resid	225	and	name	H3')	3.4	1.6	1.6
assign	(resid	225	and	name	H1')	(resid	225	and	name	H4')	2.9	1.1	1.1
assign	(resid	225	and	name	H1')	(resid	225	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	225	and	name	H1')	(resid	225	and	name	H2)	3.4	1.6	1.6
assign	(resid	225	and	name	H1')	(resid	225	and	name	H8)	2.9	1.1	1.1
assign	(resid	225	and	name	H2')	(resid	225	and	name	H3')	2.9	1.1	1.1
assign	(resid	225	and	name	H2')	(resid	225	and	name	H4')	3.4	1.6	1.6
assign	(resid	225	and	name	H2')	(resid	225	and	name	H8)	2.4	0.6	0.6
assign	(resid	225	and	name	H2')	(resid	225	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	225	and	name	H3')	(resid	225	and	name	H8)	3.9	2.1	2.1
assign	(resid	225	and	name	H4')	(resid	225	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	225	and	name	H4')	(resid	225	and	name	H8)	3.4	1.6	1.6
assign	(resid	225	and	name	H5'*)	(resid	225	and	name	H8)	3.9	2.1	2.1

assign	(resid	225	and	name	H1')	(resid	226	and	name	H8)	3.9	2.1	2.1
assign	(resid	225	and	name	H2')	(resid	226	and	name	H2')	3.4	1.6	1.6
assign	(resid	225	and	name	H2')	(resid	226	and	name	H3')	3.4	1.6	1.6
assign	(resid	225	and	name	H2')	(resid	226	and	name	H8)	3.4	1.6	1.6
assign	(resid	225	and	name	H3')	(resid	226	and	name	H8)	3.4	1.6	1.6
assign	(resid	225	and	name	H4')	(resid	226	and	name	H8)	2.9	1.1	1.1
assign	(resid	225	and	name	H8)	(resid	226	and	name	H8)	3.9	2.1	2.1

!G26

assign	(resid	226	and	name	H1')	(resid	226	and	name	H2')	2.4	0.6	0.6
assign	(resid	226	and	name	H1')	(resid	226	and	name	H3')	3.4	1.6	1.6

assign	(resid	226	and	name	H1')	(resid	226	and	name	H4')	2.9	1.1	1.1
assign	(resid	226	and	name	H1')	(resid	226	and	name	H8)	2.9	1.1	1.1
assign	(resid	226	and	name	H2')	(resid	226	and	name	H3')	2.9	1.1	1.1
assign	(resid	226	and	name	H2')	(resid	226	and	name	H8)	2.4	0.6	0.6
assign	(resid	226	and	name	H3')	(resid	226	and	name	H4')	3.4	1.6	1.6
assign	(resid	226	and	name	H3')	(resid	226	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	226	and	name	H3')	(resid	226	and	name	H8)	2.9	1.1	1.1
assign	(resid	226	and	name	H4')	(resid	226	and	name	H8)	3.9	2.1	2.1
assign	(resid	226	and	name	H5')	(resid	226	and	name	H8)	3.9	2.1	2.1
assign	(resid	226	and	name	H1')	(resid	227	and	name	H5'*)	3.9	2.1	2.1
assign	(resid	226	and	name	H1')	(resid	227	and	name	H8)	3.4	1.6	1.6
assign	(resid	226	and	name	H4')	(resid	227	and	name	H8)	3.4	1.6	1.6

!A27

assign	(resid	227	and	name	H1')	(resid	227	and	name	H2)	3.4	1.6	1.6
assign	(resid	227	and	name	H1')	(resid	227	and	name	H8)	3.4	1.6	1.6
assign	(resid	227	and	name	H1')	(resid	227	and	name	H2')	2.9	1.1	1.1
assign	(resid	227	and	name	H1')	(resid	227	and	name	H3')	3.4	1.6	1.6
assign	(resid	227	and	name	H1')	(resid	227	and	name	H4')	2.9	1.1	1.1
assign	(resid	227	and	name	H2')	(resid	227	and	name	H8)	3.4	1.6	1.6
assign	(resid	227	and	name	H3')	(resid	227	and	name	H8)	2.9	1.1	1.1
assign	(resid	227	and	name	H1')	(resid	228	and	name	H6)	4.4	2.6	2.6
assign	(resid	227	and	name	H2')	(resid	228	and	name	H6)	3.4	1.6	1.6
assign	(resid	227	and	name	H3')	(resid	228	and	name	H6)	3.4	1.6	1.6
assign	(resid	227	and	name	H8)	(resid	228	and	name	H6)	3.9	2.1	2.1

!U28

assign	(resid	228	and	name	H1')	(resid	228	and	name	H2')	3.4	1.6	1.6
assign	(resid	228	and	name	H1')	(resid	228	and	name	H3')	3.4	1.6	1.6
assign	(resid	228	and	name	H1')	(resid	228	and	name	H4')	3.4	1.6	1.6
assign	(resid	228	and	name	H1')	(resid	228	and	name	H6)	3.4	1.6	1.6
assign	(resid	228	and	name	H2')	(resid	228	and	name	H6)	3.9	2.1	2.1
assign	(resid	228	and	name	H4')	(resid	228	and	name	H6)	3.9	2.1	2.1
assign	(resid	228	and	name	H5')	(resid	228	and	name	H6)	3.9	2.1	2.1
assign	(resid	228	and	name	H1')	(resid	229	and	name	H8)	3.9	2.1	2.1
assign	(resid	228	and	name	H6)	(resid	229	and	name	H8)	3.4	1.6	1.6

!G29

assign	(resid	229	and	name	H1')	(resid	229	and	name	H2')	2.4	0.6	0.6
assign	(resid	229	and	name	H1')	(resid	229	and	name	H3')	2.9	1.1	1.1
assign	(resid	229	and	name	H1')	(resid	229	and	name	H4')	2.4	0.6	0.6
assign	(resid	229	and	name	H1')	(resid	229	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	229	and	name	H1')	(resid	229	and	name	H8)	2.9	1.1	1.1
assign	(resid	229	and	name	H2')	(resid	229	and	name	H3')	2.4	0.6	0.6
assign	(resid	229	and	name	H2')	(resid	229	and	name	H8)	3.4	1.6	1.6
assign	(resid	229	and	name	H3')	(resid	229	and	name	H4')	2.4	0.6	0.6
assign	(resid	229	and	name	H3')	(resid	229	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	229	and	name	H3')	(resid	229	and	name	H8)	2.4	0.6	0.6
assign	(resid	229	and	name	H4')	(resid	229	and	name	H8)	3.4	1.6	1.6
assign	(resid	229	and	name	H4')	(resid	229	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	229	and	name	H5'*)	(resid	229	and	name	H8)	2.9	1.1	1.1
assign	(resid	229	and	name	H1')	(resid	230	and	name	H1')	3.9	2.1	2.1
assign	(resid	229	and	name	H1')	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	229	and	name	H2')	(resid	230	and	name	H8)	2.4	0.6	0.6
assign	(resid	229	and	name	H3')	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	229	and	name	H8)	(resid	230	and	name	H8)	3.4	1.6	1.6

!G30

assign	(resid	230	and	name	H1')	(resid	230	and	name	H2')	2.4	0.6	0.6
assign	(resid	230	and	name	H1')	(resid	230	and	name	H3')	2.9	1.1	1.1
assign	(resid	230	and	name	H1')	(resid	230	and	name	H4')	2.4	0.6	0.6
assign	(resid	230	and	name	H1')	(resid	230	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	230	and	name	H1')	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	230	and	name	H2')	(resid	230	and	name	H3')	2.4	0.6	0.6
assign	(resid	230	and	name	H2')	(resid	230	and	name	H4')	3.4	1.6	1.6
assign	(resid	230	and	name	H2')	(resid	230	and	name	H8)	3.4	1.6	1.6
assign	(resid	230	and	name	H3')	(resid	230	and	name	H4')	2.4	0.6	0.6
assign	(resid	230	and	name	H3')	(resid	230	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	230	and	name	H3')	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	230	and	name	H4')	(resid	230	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	230	and	name	H4')	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	230	and	name	H5'*)	(resid	230	and	name	H8)	2.9	1.1	1.1
assign	(resid	230	and	name	H1')	(resid	231	and	name	H1')	3.9	2.1	2.1
assign	(resid	230	and	name	H1')	(resid	231	and	name	H6)	3.4	1.6	1.6
assign	(resid	230	and	name	H2')	(resid	231	and	name	H6)	2.4	0.6	0.6
assign	(resid	230	and	name	H2')	(resid	231	and	name	H5)	2.9	1.1	1.1

assign	(resid	230	and	name	H3')	(resid	231	and	name	H6)	2.9	1.1	1.1
assign	(resid	230	and	name	H3')	(resid	231	and	name	H5)	3.4	1.6	1.6
assign	(resid	230	and	name	H8)	(resid	231	and	name	H6)	3.4	1.6	1.6

!U31

assign	(resid	231	and	name	H1')	(resid	231	and	name	H2')	2.4	0.6	0.6
assign	(resid	231	and	name	H1')	(resid	231	and	name	H3')	2.9	1.1	1.1
assign	(resid	231	and	name	H1')	(resid	231	and	name	H4')	2.9	1.1	1.1
assign	(resid	231	and	name	H1')	(resid	231	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	231	and	name	H1')	(resid	231	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H1')	(resid	231	and	name	H6)	2.9	1.1	1.1
assign	(resid	231	and	name	H2')	(resid	231	and	name	H3')	2.4	0.6	0.6
assign	(resid	231	and	name	H2')	(resid	231	and	name	H4')	2.9	1.1	1.1
assign	(resid	231	and	name	H2')	(resid	231	and	name	H6)	3.4	1.6	1.6
assign	(resid	231	and	name	H2')	(resid	231	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H3')	(resid	231	and	name	H6)	2.4	0.6	0.6
assign	(resid	231	and	name	H3')	(resid	231	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H4')	(resid	231	and	name	H6)	3.4	1.6	1.6
assign	(resid	231	and	name	H4')	(resid	231	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	231	and	name	H5'*)	(resid	231	and	name	H6)	2.9	1.1	1.1
assign	(resid	231	and	name	H5'*)	(resid	231	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H5)	(resid	231	and	name	H6)	2.4	0.6	0.6
assign	(resid	231	and	name	H1')	(resid	232	and	name	H1')	3.9	2.1	2.1
assign	(resid	231	and	name	H1')	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H1')	(resid	232	and	name	H6)	2.9	1.1	1.1
assign	(resid	231	and	name	H2')	(resid	232	and	name	H6)	2.4	0.6	0.6
assign	(resid	231	and	name	H2')	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H3')	(resid	232	and	name	H6)	2.9	1.1	1.1
assign	(resid	231	and	name	H3')	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	231	and	name	H6)	(resid	232	and	name	H6)	3.9	2.1	2.1

!C32

assign	(resid	232	and	name	H1')	(resid	232	and	name	H2')	2.4	0.6	0.6
assign	(resid	232	and	name	H1')	(resid	232	and	name	H3')	2.9	1.1	1.1
assign	(resid	232	and	name	H1')	(resid	232	and	name	H4')	2.9	1.1	1.1
assign	(resid	232	and	name	H1')	(resid	232	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	232	and	name	H1')	(resid	232	and	name	H6)	2.9	1.1	1.1
assign	(resid	232	and	name	H2')	(resid	232	and	name	H6)	3.4	1.6	1.6
assign	(resid	232	and	name	H3')	(resid	232	and	name	H6)	2.9	1.1	1.1
assign	(resid	232	and	name	H5'*)	(resid	232	and	name	H6)	2.9	1.1	1.1
assign	(resid	232	and	name	H5'*)	(resid	232	and	name	H5)	3.4	1.6	1.6
assign	(resid	232	and	name	H5)	(resid	232	and	name	H6)	2.4	0.6	0.6
assign	(resid	232	and	name	H1')	(resid	233	and	name	H1')	3.9	2.1	2.1
assign	(resid	232	and	name	H1')	(resid	233	and	name	H6)	3.4	1.6	1.6
assign	(resid	232	and	name	H2')	(resid	233	and	name	H6)	2.4	0.6	0.6
assign	(resid	232	and	name	H2')	(resid	233	and	name	H5)	3.4	1.6	1.6
assign	(resid	232	and	name	H3')	(resid	233	and	name	H6)	2.9	1.1	1.1
assign	(resid	232	and	name	H3')	(resid	233	and	name	H5)	3.4	1.6	1.6

!C33

assign	(resid	233	and	name	H1')	(resid	233	and	name	H2')	2.4	0.6	0.6
assign	(resid	233	and	name	H1')	(resid	233	and	name	H3')	2.9	1.1	1.1
assign	(resid	233	and	name	H1')	(resid	233	and	name	H4')	2.9	1.1	1.1
assign	(resid	233	and	name	H1')	(resid	233	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	233	and	name	H1')	(resid	233	and	name	H6)	2.9	1.1	1.1
assign	(resid	233	and	name	H2')	(resid	233	and	name	H3')	2.4	0.6	0.6
assign	(resid	233	and	name	H2')	(resid	233	and	name	H5)	3.9	2.1	2.1
assign	(resid	233	and	name	H2')	(resid	233	and	name	H6)	2.9	1.1	1.1
assign	(resid	233	and	name	H3')	(resid	233	and	name	H4')	2.4	0.6	0.6
assign	(resid	233	and	name	H3')	(resid	233	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	233	and	name	H3')	(resid	233	and	name	H5)	3.4	1.6	1.6
assign	(resid	233	and	name	H3')	(resid	233	and	name	H6)	2.4	0.6	0.6
assign	(resid	233	and	name	H4')	(resid	233	and	name	H6)	2.9	1.1	1.1
assign	(resid	233	and	name	H4')	(resid	233	and	name	H5'*)	2.4	0.6	0.6
assign	(resid	233	and	name	H5'*)	(resid	233	and	name	H6)	2.9	1.1	1.1
assign	(resid	233	and	name	H5'*)	(resid	233	and	name	H5)	3.4	1.6	1.6
assign	(resid	233	and	name	H5)	(resid	233	and	name	H6)	2.4	0.6	0.6

set echo = on end
set message = on end

```
!complex restraints L30NP_noe.tbl
set echo = off end
set message = off end
!RNA to protein NOE
```

assign	(resid	223	and	name	H2)	(resid	85	and	name	HB*)	3.9	2.1	2.1
assign	(resid	223	and	name	H2)	(resid	85	and	name	HD*)	3.4	1.6	1.6
assign	(resid	223	and	name	H2)	(resid	85	and	name	HE*)	3.9	2.1	2.1
assign	(resid	223	and	name	H2)	(resid	85	and	name	HZ)	3.9	2.1	2.1
assign	(resid	223	and	name	H2')	(resid	85	and	name	HE*)	3.9	2.1	2.1
assign	(resid	224	and	name	H1')	(resid	25	and	name	HB1)	2.9	1.1	1.1
assign	(resid	224	and	name	H1')	(resid	25	and	name	HB2)	3.4	1.6	1.6
assign	(resid	224	and	name	H1')	(resid	87	and	name	HB)	3.9	2.1	2.1
assign	(resid	224	and	name	H1')	(resid	26	and	name	HA*)	2.9	1.1	1.1
assign	(resid	224	and	name	H1')	(resid	85	and	name	HE1)	3.4	1.6	1.6
assign	(resid	224	and	name	H2')	(resid	85	and	name	HE1)	3.4	1.6	1.6
assign	(resid	224	and	name	H2')	(resid	85	and	name	HD1)	2.9	1.1	1.1
assign	(resid	224	and	name	H3')	(resid	85	and	name	HD*)	3.9	2.1	2.1
assign	(resid	224	and	name	H8)	(resid	25	and	name	HA)	3.4	1.6	1.6
assign	(resid	224	and	name	H8)	(resid	25	and	name	HB1)	2.9	1.1	1.1
assign	(resid	224	and	name	H8)	(resid	25	and	name	HB2)	3.4	1.6	1.6
assign	(resid	224	and	name	H8)	(resid	25	and	name	HD1*)	3.4	1.6	1.6
assign	(resid	224	and	name	H8)	(resid	26	and	name	HA1)	2.9	1.1	1.1
assign	(resid	224	and	name	H8)	(resid	26	and	name	HA2)	2.9	1.1	1.1
assign	(resid	224	and	name	H8)	(resid	85	and	name	HZ)	3.9	2.1	2.1
assign	(resid	224	and	name	H8)	(resid	85	and	name	HE1)	3.9	2.1	2.1
assign	(resid	224	and	name	H8)	(resid	87	and	name	HG1*)	3.9	2.1	2.1
assign	(resid	224	and	name	H8)	(resid	87	and	name	HG2*)	3.9	2.1	2.1
assign	(resid	225	and	name	H1')	(resid	52	and	name	HG*)	3.4	1.6	1.6
assign	(resid	225	and	name	H1')	(resid	52	and	name	HD*)	3.4	1.6	1.6
assign	(resid	225	and	name	H1')	(resid	49	and	name	HD*)	3.9	2.1	2.1
assign	(resid	225	and	name	H4')	(resid	27	and	name	HB*)	3.4	1.6	1.6
assign	(resid	225	and	name	H4')	(resid	27	and	name	HD*)	3.4	1.6	1.6
assign	(resid	225	and	name	H4')	(resid	52	and	name	HG*)	3.9	2.1	2.1
assign	(resid	225	and	name	H4')	(resid	52	and	name	HD*)	3.9	2.1	2.1
assign	(resid	225	and	name	H4')	(resid	52	and	name	HE*)	3.4	1.6	1.6
assign	(resid	225	and	name	H5'*)	(resid	27	and	name	HD*)	3.9	2.1	2.1
assign	(resid	225	and	name	H5'*)	(resid	27	and	name	HB*)	3.9	2.1	2.1
assign	(resid	225	and	name	H2)	(resid	48	and	name	HA)	3.4	1.6	1.6
assign	(resid	225	and	name	H2)	(resid	48	and	name	HG2*)	3.4	1.6	1.6
assign	(resid	225	and	name	H2)	(resid	49	and	name	HD*)	3.9	2.1	2.1
assign	(resid	225	and	name	H2)	(resid	49	and	name	HG*)	3.9	2.1	2.1
assign	(resid	225	and	name	H2)	(resid	49	and	name	HB*)	3.4	1.6	1.6
assign	(resid	225	and	name	H8)	(resid	52	and	name	HG*)	3.9	2.1	2.1
assign	(resid	225	and	name	H8)	(resid	52	and	name	HD*)	3.9	2.1	2.1
assign	(resid	225	and	name	H8)	(resid	27	and	name	HD2)	3.9	2.1	2.1
assign	(resid	226	and	name	H1')	(resid	28	and	name	HG*)	3.4	1.6	1.6
assign	(resid	226	and	name	H1')	(resid	28	and	name	HD*)	3.9	2.1	2.1
assign	(resid	226	and	name	H1')	(resid	28	and	name	HE*)	3.9	2.1	2.1
assign	(resid	226	and	name	H2')	(resid	28	and	name	HG*)	3.9	2.1	2.1
assign	(resid	226	and	name	H2')	(resid	28	and	name	HD*)	3.9	2.1	2.1
assign	(resid	226	and	name	H2')	(resid	28	and	name	HE*)	3.9	2.1	2.1
assign	(resid	226	and	name	H8)	(resid	27	and	name	HA)	3.9	2.1	2.1
assign	(resid	226	and	name	H8)	(resid	27	and	name	HB*)	3.9	2.1	2.1
assign	(resid	226	and	name	H8)	(resid	27	and	name	HD2)	3.4	1.6	1.6
assign	(resid	226	and	name	H8)	(resid	27	and	name	HE2)	3.9	2.1	2.1
assign	(resid	226	and	name	H8)	(resid	28	and	name	HA)	3.9	2.1	2.1
assign	(resid	226	and	name	H8)	(resid	28	and	name	HG*)	3.4	1.6	1.6
assign	(resid	226	and	name	H8)	(resid	28	and	name	HB*)	3.4	1.6	1.6
assign	(resid	226	and	name	H8)	(resid	52	and	name	HG*)	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	29	and	name	HN)	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	29	and	name	HB#)	3.9	2.1	2.1
assign	(resid	207	and	name	H1)	(resid	26	and	name	HN)	3.9	2.1	2.1
assign	(resid	224	and	name	H1)	(resid	85	and	name	HB*)	3.9	2.1	2.1
assign	(resid	224	and	name	H1)	(resid	85	and	name	HZ)	3.9	2.1	2.1
assign	(resid	224	and	name	H1)	(resid	85	and	name	HD2)	3.4	1.6	1.6
assign	(resid	224	and	name	H1)	(resid	85	and	name	HE2)	3.4	1.6	1.6

```
!protein exchangeable to RNA restraints
```

assign	(resid	25	and	name	HN)	(resid	224	and	name	H8)	3.9	2.1	2.1
assign	(resid	26	and	name	HN)	(resid	224	and	name	H1')	2.9	1.1	1.1
assign	(resid	26	and	name	HN)	(resid	224	and	name	H8)	3.4	1.6	1.6
assign	(resid	26	and	name	HN)	(resid	225	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	27	and	name	HN)	(resid	224	and	name	H1')	3.9	2.1	2.1
assign	(resid	27	and	name	HN)	(resid	224	and	name	H8)	3.9	2.1	2.1
assign	(resid	27	and	name	HN)	(resid	225	and	name	H4')	3.4	1.6	1.6
assign	(resid	27	and	name	HN)	(resid	225	and	name	H5'*)	3.4	1.6	1.6
assign	(resid	27	and	name	HN)	(resid	226	and	name	H5'*)	2.9	1.1	1.1
assign	(resid	27	and	name	HN)	(resid	226	and	name	H8)	3.9	2.1	2.1

```
assign  (resid  28   and   name   HN )  (resid  225   and   name   H4' )   3.9   2.1   2.1
assign  (resid  28   and   name   HN )  (resid  226   and   name   H5'*)   3.4   1.6   1.6
assign  (resid  28   and   name   HN )  (resid  226   and   name   H8 )    3.4   1.6   1.6
assign  (resid  29   and   name   HN )  (resid  226   and   name   H4' )   2.9   1.1   1.1
assign  (resid  52   and   name   HE )  (resid  225   and   name   H1' )   2.9   1.1   1.1
assign  (resid  52   and   name   HE )  (resid  226   and   name   H2' )   3.4   1.6   1.6
assign  (resid  88   and   name   HN )  (resid  224   and   name   H3' )   3.9   2.1   2.1

set echo = on end
set message = on end
```

```
restraint dihedra reset
set echo = off end
set message = off end
!protein dihedral restraints L30P_dih.tbl
!backbond phi dihedar angles derived from HNHA experiment
!! Phi restraints minimum deviation = 30 degrees
!1st numbers is energy contants, 2nd is the degree of dihedral angle, 3rd is the rang and 4th the exponential function
```

assign	(resid	3	and	name	C)	(resid	4	and	name	N)			
	(resid	4	and	name	CA)	(resid	4	and	name	C)	1	-73	30	2
assign	(resid	4	and	name	C)	(resid	5	and	name	N)			
	(resid	5	and	name	CA)	(resid	5	and	name	C)	1	-71	30	2
assign	(resid	5	and	name	C)	(resid	6	and	name	N)			
	(resid	6	and	name	CA)	(resid	6	and	name	C)	1	-77	30	2
assign	(resid	6	and	name	C)	(resid	7	and	name	N)			
	(resid	7	and	name	CA)	(resid	7	and	name	C)	1	-72	30	2
assign	(resid	7	and	name	C)	(resid	8	and	name	N)			
	(resid	8	and	name	CA)	(resid	8	and	name	C)	1	-73	30	2
assign	(resid	8	and	name	C)	(resid	9	and	name	N)			
	(resid	9	and	name	CA)	(resid	9	and	name	C)	1	-73	30	2
assign	(resid	9	and	name	C)	(resid	10	and	name	N)			
	(resid	10	and	name	CA)	(resid	10	and	name	C)	1	-53	30	2
assign	(resid	11	and	name	C)	(resid	12	and	name	N)			
	(resid	12	and	name	CA)	(resid	12	and	name	C)	1	-59	30	2
assign	(resid	13	and	name	C)	(resid	14	and	name	N)			
	(resid	14	and	name	CA)	(resid	14	and	name	C)	1	-65	30	2
assign	(resid	14	and	name	C)	(resid	15	and	name	N)			
	(resid	15	and	name	CA)	(resid	15	and	name	C)	1	-58	30	2
assign	(resid	15	and	name	C)	(resid	16	and	name	N)			
	(resid	16	and	name	CA)	(resid	16	and	name	C)	1	-60	30	2
assign	(resid	16	and	name	C)	(resid	17	and	name	N)			
	(resid	17	and	name	CA)	(resid	17	and	name	C)	1	-60	30	2
assign	(resid	17	and	name	C)	(resid	18	and	name	N)			
	(resid	18	and	name	CA)	(resid	18	and	name	C)	1	-59	30	2
assign	(resid	18	and	name	C)	(resid	19	and	name	N)			
	(resid	19	and	name	CA)	(resid	19	and	name	C)	1	-58	30	2
assign	(resid	19	and	name	C)	(resid	20	and	name	N)			
	(resid	20	and	name	CA)	(resid	20	and	name	C)	1	-125	33	2
assign	(resid	21	and	name	C)	(resid	22	and	name	N)			
	(resid	22	and	name	CA)	(resid	22	and	name	C)	1	-120	31	2
assign	(resid	22	and	name	C)	(resid	23	and	name	N)			
	(resid	23	and	name	CA)	(resid	23	and	name	C)	1	-119	55	2
assign	(resid	23	and	name	C)	(resid	24	and	name	N)			
	(resid	24	and	name	CA)	(resid	24	and	name	C)	1	-120	48	2
assign	(resid	24	and	name	C)	(resid	25	and	name	N)			
	(resid	25	and	name	CA)	(resid	25	and	name	C)	1	-119	42	2
assign	(resid	29	and	name	C)	(resid	30	and	name	N)			
	(resid	30	and	name	CA)	(resid	30	and	name	C)	1	-66	30	2
assign	(resid	31	and	name	C)	(resid	32	and	name	N)			
	(resid	32	and	name	CA)	(resid	32	and	name	C)	1	-61	30	2
assign	(resid	32	and	name	C)	(resid	33	and	name	N)			
	(resid	33	and	name	CA)	(resid	33	and	name	C)	1	-69	30	2
assign	(resid	33	and	name	C)	(resid	34	and	name	N)			
	(resid	34	and	name	CA)	(resid	34	and	name	C)	1	-67	30	2
assign	(resid	34	and	name	C)	(resid	35	and	name	N)			
	(resid	35	and	name	CA)	(resid	35	and	name	C)	1	-57	30	2
assign	(resid	37	and	name	C)	(resid	38	and	name	N)			
	(resid	38	and	name	CA)	(resid	38	and	name	C)	1	-37	30	2
assign	(resid	38	and	name	C)	(resid	39	and	name	N)			
	(resid	39	and	name	CA)	(resid	39	and	name	C)	1	-64	30	2
assign	(resid	40	and	name	C)	(resid	41	and	name	N)			
	(resid	41	and	name	CA)	(resid	41	and	name	C)	1	-67	30	2
assign	(resid	44	and	name	C)	(resid	45	and	name	N)			
	(resid	45	and	name	CA)	(resid	45	and	name	C)	1	-120	46	2
assign	(resid	45	and	name	C)	(resid	46	and	name	N)			
	(resid	46	and	name	CA)	(resid	46	and	name	C)	1	-59	46.5	2
assign	(resid	47	and	name	C)	(resid	48	and	name	N)			
	(resid	48	and	name	CA)	(resid	48	and	name	C)	1	-51	35	2
assign	(resid	50	and	name	C)	(resid	51	and	name	N)			
	(resid	51	and	name	CA)	(resid	51	and	name	C)	1	-54	35	2
assign	(resid	51	and	name	C)	(resid	52	and	name	N)			
	(resid	52	and	name	CA)	(resid	52	and	name	C)	1	-69	35	2
assign	(resid	52	and	name	C)	(resid	53	and	name	N)			
	(resid	53	and	name	CA)	(resid	53	and	name	C)	1	-54	35	2
assign	(resid	53	and	name	C)	(resid	54	and	name	N)			
	(resid	54	and	name	CA)	(resid	54	and	name	C)	1	-57	35	2
assign	(resid	54	and	name	C)	(resid	55	and	name	N)			
	(resid	55	and	name	CA)	(resid	55	and	name	C)	1	-58	35	2

```
assign (resid  55 and name C ) (resid  56 and name N )
       (resid  56 and name CA) (resid  56 and name C )   1   -65   35   2
assign (resid  56 and name C ) (resid  57 and name N )
       (resid  57 and name CA) (resid  57 and name C )   1   -62   35   2
assign (resid  57 and name C ) (resid  58 and name N )
       (resid  58 and name CA) (resid  58 and name C )   1   -54   30   2
assign (resid  59 and name C ) (resid  60 and name N )
       (resid  60 and name CA) (resid  60 and name C )   1   -54   30   2
assign (resid  60 and name C ) (resid  61 and name N )
       (resid  61 and name CA) (resid  61 and name C )   1   -62   30   2
assign (resid  61 and name C ) (resid  62 and name N )
       (resid  62 and name CA) (resid  62 and name C )   1   -63   30   2
assign (resid  62 and name C ) (resid  63 and name N )
       (resid  63 and name CA) (resid  63 and name C )   1   -79   30   2
assign (resid  65 and name C ) (resid  66 and name N )
       (resid  66 and name CA) (resid  66 and name C )   1  -118   59   2
assign (resid  66 and name C ) (resid  67 and name N )
       (resid  67 and name CA) (resid  67 and name C )   1  -120   32   2
assign (resid  67 and name C ) (resid  68 and name N )
       (resid  68 and name CA) (resid  68 and name C )   1  -120   36   2
assign (resid  68 and name C ) (resid  69 and name N )
       (resid  69 and name CA) (resid  69 and name C )   1  -120   45   2
assign (resid  69 and name C ) (resid  70 and name N )
       (resid  70 and name CA) (resid  70 and name C )   1  -120   57   2
assign (resid  70 and name C ) (resid  71 and name N )
       (resid  71 and name CA) (resid  71 and name C )   1  -120   51   2
assign (resid  72 and name C ) (resid  73 and name N )
       (resid  73 and name CA) (resid  73 and name C )   1   -58   30   2
assign (resid  73 and name C ) (resid  74 and name N )
       (resid  74 and name CA) (resid  74 and name C )   1   -65   30   2
assign (resid  74 and name C ) (resid  75 and name N )
       (resid  75 and name CA) (resid  75 and name C )   1   -80   30   2
assign (resid  75 and name C ) (resid  76 and name N )
       (resid  76 and name CA) (resid  76 and name C )   1   -71   30   2
assign (resid  76 and name C ) (resid  77 and name N )
       (resid  77 and name CA) (resid  77 and name C )   1   -64   30   2
assign (resid  77 and name C ) (resid  78 and name N )
       (resid  78 and name CA) (resid  78 and name C )   1   -68   30   2
assign (resid  79 and name C ) (resid  80 and name N )
       (resid  80 and name CA) (resid  80 and name C )   1   -70   30   2
assign (resid  80 and name C ) (resid  81 and name N )
       (resid  81 and name CA) (resid  81 and name C )   1   -70   30   2
assign (resid  82 and name C ) (resid  83 and name N )
       (resid  83 and name CA) (resid  83 and name C )   1   -73   30   2
assign (resid  83 and name C ) (resid  84 and name N )
       (resid  84 and name CA) (resid  84 and name C )   1   -77   30   2
assign (resid  84 and name C ) (resid  85 and name N )
       (resid  85 and name CA) (resid  85 and name C )   1   -66   30   2
assign (resid  85 and name C ) (resid  86 and name N )
       (resid  86 and name CA) (resid  86 and name C )   1   -96   30   2
assign (resid  88 and name C ) (resid  89 and name N )
       (resid  89 and name CA) (resid  89 and name C )   1   -60   30   2
assign (resid  90 and name C ) (resid  91 and name N )
       (resid  91 and name CA) (resid  91 and name C )   1  -120   46   2
assign (resid  91 and name C ) (resid  92 and name N )
       (resid  92 and name CA) (resid  92 and name C )   1  -120   34   2
assign (resid  92 and name C ) (resid  93 and name N )
       (resid  93 and name CA) (resid  93 and name C )   1  -120   34   2
assign (resid  93 and name C ) (resid  94 and name N )
       (resid  94 and name CA) (resid  94 and name C )   1   -72   30   2
assign (resid  94 and name C ) (resid  95 and name N )
       (resid  95 and name CA) (resid  95 and name C )   1   -70   30   2
assign (resid  95 and name C ) (resid  96 and name N )
       (resid  96 and name CA) (resid  96 and name C )   1   -59   30   2
assign (resid  96 and name C ) (resid  97 and name N )
       (resid  97 and name CA) (resid  97 and name C )   1  -120   36   2
assign (resid  97 and name C ) (resid  98 and name N )
       (resid  98 and name CA) (resid  98 and name C )   1   -65   30   2
assign (resid  98 and name C ) (resid  99 and name N )
       (resid  99 and name CA) (resid  99 and name C )   1  -120   42   2
assign (resid 100 and name C ) (resid 101 and name N )
       (resid 101 and name CA) (resid 101 and name C )   1   -65   30   2
assign (resid 101 and name C ) (resid 102 and name N )
       (resid 102 and name CA) (resid 102 and name C )   1  -120   39   2
assign (resid 102 and name C ) (resid 103 and name N )
       (resid 103 and name CA) (resid 103 and name C )   1   -90   30   2
assign (resid 103 and name C ) (resid 104 and name N )
```

```
              (resid  104   and   name   CA )   (resid  104   and   name   C )   1   -76   30   2
assign        (resid  104   and   name   C  )   (resid  105   and   name   N )
              (resid  105   and   name   CA )   (resid  105   and   name   C )   1   -75   30   2
!chi1 angles from the stereo-specific assignment
assign        (resid  4     and   name   N)     (resid  4     and   name   CA)
              (resid  4     and   name   CB)    (resid  4     and   name   CG1)  1   185   30   2
assign        (resid  5     and   name   N)     (resid  5     and   name   CA)
              (resid  5     and   name   CB)    (resid  5     and   name   CG)   1   60    30   2
assign        (resid  7     and   name   N)     (resid  7     and   name   CA)
              (resid  7     and   name   CB)    (resid  7     and   name   CG)   1   60    30   2
assign        (resid  9     and   name   N)     (resid  9     and   name   CA)
              (resid  9     and   name   CB)    (resid  9     and   name   OG)   1   180   30   2
assign        (resid  10    and   name   N)     (resid  10    and   name   CA)
              (resid  10    and   name   CB)    (resid  10    and   name   CG1)  1   180   30   2
assign        (resid  12    and   name   N)     (resid  12    and   name   CA)
              (resid  12    and   name   CB)    (resid  12    and   name   CG)   1   180   30   2
assign        (resid  14    and   name   N)     (resid  14    and   name   CA)
              (resid  14    and   name   CB)    (resid  14    and   name   CG)   1   180   30   2
assign        (resid  16    and   name   N)     (resid  16    and   name   CA)
              (resid  16    and   name   CB)    (resid  16    and   name   CG)   1   180   30   2
assign        (resid  17    and   name   N)     (resid  17    and   name   CA)
              (resid  17    and   name   CB)    (resid  17    and   name   CG1)  1   180   30   2
assign        (resid  18    and   name   N)     (resid  18    and   name   CA)
              (resid  18    and   name   CB)    (resid  18    and   name   CG1)  1   -60   30   2
assign        (resid  19    and   name   N)     (resid  19    and   name   CA)
              (resid  19    and   name   CB)    (resid  19    and   name   CG)   1   60    30   2
assign        (resid  20    and   name   N)     (resid  20    and   name   CA)
              (resid  20    and   name   CB)    (resid  20    and   name   OG)   1   60    30   2
assign        (resid  22    and   name   N)     (resid  22    and   name   CA)
              (resid  22    and   name   CB)    (resid  22    and   name   CG)   1   -60   30   2
assign        (resid  23    and   name   N)     (resid  23    and   name   CA)
              (resid  23    and   name   CB)    (resid  23    and   name   CG)   1   60    30   2
assign        (resid  24    and   name   N)     (resid  24    and   name   CA)
              (resid  24    and   name   CB)    (resid  24    and   name   OG1)  1   180   20   2
assign        (resid  25    and   name   N)     (resid  25    and   name   CA)
              (resid  25    and   name   CB)    (resid  25    and   name   CG)   1   -60   30   2
assign        (resid  30    and   name   N)     (resid  30    and   name   CA)
              (resid  30    and   name   CB)    (resid  30    and   name   OG1)  1   180   20   2
assign        (resid  31    and   name   N)     (resid  31    and   name   CA)
              (resid  31    and   name   CB)    (resid  31    and   name   CG1)  1   185   30   2
assign        (resid  33    and   name   N)     (resid  33    and   name   CA)
              (resid  33    and   name   CB)    (resid  33    and   name   OG)   1   -60   30   2
assign        (resid  34    and   name   N)     (resid  34    and   name   CA)
              (resid  34    and   name   CB)    (resid  34    and   name   CG)   1   -60   30   2
assign        (resid  35    and   name   N)     (resid  35    and   name   CA)
              (resid  35    and   name   CB)    (resid  35    and   name   CG)   1   60    30   2
assign        (resid  36    and   name   N)     (resid  36    and   name   CA)
              (resid  36    and   name   CB)    (resid  36    and   name   CG)   1   -60   30   2
assign        (resid  39    and   name   N)     (resid  39    and   name   CA)
              (resid  39    and   name   CB)    (resid  39    and   name   OG)   1   60    30   2
assign        (resid  42    and   name   N)     (resid  42    and   name   CA)
              (resid  42    and   name   CB)    (resid  42    and   name   CG1)  1   60    30   2
assign        (resid  43    and   name   N)     (resid  43    and   name   CA)
              (resid  43    and   name   CB)    (resid  43    and   name   CG1)  1   -60   30   2
assign        (resid  44    and   name   N)     (resid  44    and   name   CA)
              (resid  44    and   name   CB)    (resid  44    and   name   CG1)  1   60    30   2
assign        (resid  47    and   name   N)     (resid  47    and   name   CA)
              (resid  47    and   name   CB)    (resid  47    and   name   CG)   1   180   30   2
assign        (resid  48    and   name   N)     (resid  48    and   name   CA)
              (resid  48    and   name   CB)    (resid  48    and   name   OG1)  1   180   20   2
assign        (resid  50    and   name   N)     (resid  50    and   name   CA)
              (resid  50    and   name   CB)    (resid  50    and   name   CG1)  1   185   30   2
assign        (resid  51    and   name   N)     (resid  51    and   name   CA)
              (resid  51    and   name   CB)    (resid  51    and   name   CG)   1   -60   30   2
assign        (resid  52    and   name   N)     (resid  52    and   name   CA)
              (resid  52    and   name   CB)    (resid  52    and   name   CG)   1   180   30   2
assign        (resid  53    and   name   N)     (resid  53    and   name   CA)
              (resid  53    and   name   CB)    (resid  53    and   name   CG)   1   60    30   2
assign        (resid  57    and   name   N)     (resid  57    and   name   CA)
              (resid  57    and   name   CB)    (resid  57    and   name   CG)   1   180   30   2
assign        (resid  58    and   name   N)     (resid  58    and   name   CA)
              (resid  58    and   name   CB)    (resid  58    and   name   CG)   1   180   30   2
assign        (resid  59    and   name   N)     (resid  59    and   name   CA)
              (resid  59    and   name   CB)    (resid  59    and   name   CG)   1   -60   30   2
assign        (resid  61    and   name   N)     (resid  61    and   name   CA)
              (resid  61    and   name   CB)    (resid  61    and   name   CG)   1   180   30   2
assign        (resid  62    and   name   N)     (resid  62    and   name   CA)
```

	(resid	62	and	name	CB)	(resid	62	and	name	CG)	1	-60	30	2
assign	(resid	65	and	name	N)	(resid	65	and	name	CA)				
	(resid	65	and	name	CB)	(resid	65	and	name	OG1)	1	-60	20	2
assign	(resid	67	and	name	N)	(resid	67	and	name	CA)				
	(resid	67	and	name	CB)	(resid	67	and	name	CG1)	1	180	30	2
assign	(resid	68	and	name	N)	(resid	68	and	name	CA)				
	(resid	68	and	name	CB)	(resid	68	and	name	CG)	1	180	30	2
assign	(resid	69	and	name	N)	(resid	69	and	name	CA)				
	(resid	69	and	name	CB)	(resid	69	and	name	CG)	1	-60	30	2
assign	(resid	70	and	name	N)	(resid	70	and	name	CA)				
	(resid	70	and	name	CB)	(resid	70	and	name	CG)	1	180	30	2
assign	(resid	71	and	name	N)	(resid	71	and	name	CA)				
	(resid	71	and	name	CB)	(resid	71	and	name	CG)	1	180	30	2
assign	(resid	74	and	name	N)	(resid	74	and	name	CA)				
	(resid	74	and	name	CB)	(resid	74	and	name	CG)	1	60	30	2
assign	(resid	75	and	name	N)	(resid	75	and	name	CA)				
	(resid	75	and	name	CB)	(resid	75	and	name	CG)	1	180	30	2
assign	(resid	76	and	name	N)	(resid	76	and	name	CA)				
	(resid	76	and	name	CB)	(resid	76	and	name	CG)	1	-60	30	2
assign	(resid	77	and	name	N)	(resid	77	and	name	CA)				
	(resid	77	and	name	CB)	(resid	77	and	name	CG)	1	-60	30	2
assign	(resid	79	and	name	N)	(resid	79	and	name	CA)				
	(resid	79	and	name	CB)	(resid	79	and	name	OG1)	1	-60	30	2
assign	(resid	81	and	name	N)	(resid	81	and	name	CA)				
	(resid	81	and	name	CB)	(resid	81	and	name	CG1)	1	-60	30	2
assign	(resid	85	and	name	N)	(resid	85	and	name	CA)				
	(resid	85	and	name	CB)	(resid	85	and	name	CG)	1	60	30	2
assign	(resid	87	and	name	N)	(resid	87	and	name	CA)				
	(resid	87	and	name	CB)	(resid	87	and	name	CG1)	1	60	30	2
assign	(resid	89	and	name	N)	(resid	89	and	name	CA)				
	(resid	89	and	name	CB)	(resid	89	and	name	CG1)	1	60	30	2
assign	(resid	90	and	name	N)	(resid	90	and	name	CA)				
	(resid	90	and	name	CB)	(resid	90	and	name	CG1)	1	185	30	2
assign	(resid	91	and	name	N)	(resid	91	and	name	CA)				
	(resid	91	and	name	CB)	(resid	91	and	name	OG)	1	180	30	2
assign	(resid	92	and	name	N)	(resid	92	and	name	CA)				
	(resid	92	and	name	CB)	(resid	92	and	name	CG1)	1	-60	30	2
assign	(resid	94	and	name	N)	(resid	94	and	name	CA)				
	(resid	94	and	name	CB)	(resid	94	and	name	CG)	1	60	30	2
assign	(resid	97	and	name	N)	(resid	97	and	name	CA)				
	(resid	97	and	name	CB)	(resid	97	and	name	CG)	1	180	30	2
assign	(resid	99	and	name	N)	(resid	99	and	name	CA)				
	(resid	99	and	name	CB)	(resid	99	and	name	CG)	1	60	30	2
assign	(resid	100	and	name	N)	(resid	100	and	name	CA)				
	(resid	100	and	name	CB)	(resid	100	and	name	CG1)	1	60	30	2

set echo = on end
set message = on end

restraint dihedra reset
set echo = off end
set message = off end
!RNA dihedral restraints L30N_dih.tbl
!planar restraints to keep amino protons in bases pairs from flipping
!1st number is energy constant, 2nd dihedral angle, 3rd range, and 4th exponential function

assign	(resid	201	and	name	H22)	(resid	201	and	name	N2)				
	(resid	201	and	name	C2)	(resid	201	and	name	N1)	1	0	5	2
assign	(resid	202	and	name	H22)	(resid	202	and	name	N2)				
	(resid	202	and	name	C2)	(resid	202	and	name	N1)	1	0	5	2
assign	(resid	203	and	name	H62)	(resid	203	and	name	N6)				
	(resid	203	and	name	C6)	(resid	203	and	name	N1)	1	0	5	2
assign	(resid	204	and	name	H42)	(resid	204	and	name	N4)				
	(resid	204	and	name	C4)	(resid	204	and	name	N3)	1	0	5	2
assign	(resid	205	and	name	H42)	(resid	205	and	name	N4)				
	(resid	205	and	name	C4)	(resid	205	and	name	N3)	1	0	5	2
assign	(resid	206	and	name	H22)	(resid	206	and	name	N2)				
	(resid	206	and	name	C2)	(resid	206	and	name	N1)	1	0	5	2
assign	(resid	207	and	name	H22)	(resid	207	and	name	N2)				
	(resid	207	and	name	C2)	(resid	207	and	name	N1)	1	0	5	2
assign	(resid	208	and	name	H62)	(resid	208	and	name	N6)				
	(resid	208	and	name	C6)	(resid	208	and	name	N1)	1	0	5	2
assign	(resid	209	and	name	H22)	(resid	209	and	name	N2)				
	(resid	209	and	name	C2)	(resid	209	and	name	N1)	1	0	5	2
assign	(resid	211	and	name	H22)	(resid	211	and	name	N2)				
	(resid	211	and	name	C2)	(resid	211	and	name	N1)	1	0	5	2
assign	(resid	213	and	name	H42)	(resid	213	and	name	N4)				
	(resid	213	and	name	C4)	(resid	213	and	name	N3)	1	0	5	2
assign	(resid	214	and	name	H22)	(resid	214	and	name	N2)				
	(resid	214	and	name	C2)	(resid	214	and	name	N1)	1	0	5	2
assign	(resid	215	and	name	H42)	(resid	215	and	name	N4)				
	(resid	215	and	name	C4)	(resid	215	and	name	N3)	1	0	5	2
assign	(resid	216	and	name	H62)	(resid	216	and	name	N6)				
	(resid	216	and	name	C6)	(resid	216	and	name	N1)	1	0	5	2
assign	(resid	217	and	name	H62)	(resid	217	and	name	N6)				
	(resid	217	and	name	C6)	(resid	217	and	name	N1)	1	0	5	2
assign	(resid	218	and	name	H22)	(resid	218	and	name	N2)				
	(resid	218	and	name	C2)	(resid	218	and	name	N1)	1	0	5	2
assign	(resid	219	and	name	H62)	(resid	219	and	name	N6)				
	(resid	219	and	name	C6)	(resid	219	and	name	N1)	1	0	5	2
assign	(resid	220	and	name	H42)	(resid	220	and	name	N4)				
	(resid	220	and	name	C4)	(resid	220	and	name	N3)	1	0	5	2
assign	(resid	221	and	name	H22)	(resid	221	and	name	N2)				
	(resid	221	and	name	C2)	(resid	221	and	name	N1)	1	0	5	2
assign	(resid	222	and	name	H42)	(resid	222	and	name	N4)				
	(resid	222	and	name	C4)	(resid	222	and	name	N3)	1	0	5	2
assign	(resid	223	and	name	H62)	(resid	223	and	name	N6)				
	(resid	223	and	name	C6)	(resid	223	and	name	N1)	1	0	5	2
assign	(resid	224	and	name	H22)	(resid	224	and	name	N2)				
	(resid	224	and	name	C2)	(resid	224	and	name	N1)	1	0	5	2
assign	(resid	225	and	name	H62)	(resid	225	and	name	N6)				
	(resid	225	and	name	C6)	(resid	225	and	name	N1)	1	0	5	2
assign	(resid	226	and	name	H22)	(resid	226	and	name	N2)				
	(resid	226	and	name	C2)	(resid	226	and	name	N1)	1	0	5	2
assign	(resid	227	and	name	H62)	(resid	227	and	name	N6)				
	(resid	227	and	name	C6)	(resid	227	and	name	N1)	1	0	5	2
assign	(resid	229	and	name	H22)	(resid	229	and	name	N2)				
	(resid	229	and	name	C2)	(resid	229	and	name	N1)	1	0	5	2
assign	(resid	230	and	name	H22)	(resid	230	and	name	N2)				
	(resid	230	and	name	C2)	(resid	230	and	name	N1)	1	0	5	2
assign	(resid	232	and	name	H42)	(resid	232	and	name	N4)				
	(resid	232	and	name	C4)	(resid	232	and	name	N3)	1	0	5	2
assign	(resid	233	and	name	H42)	(resid	233	and	name	N4)				
	(resid	233	and	name	C4)	(resid	233	and	name	N3)	1	0	5	2

!Glycosidic constraints

assign	(resid	201	and	name	O4')	(resid	201	and	name	C1')				
	(resid	201	and	name	N9)	(resid	201	and	name	C4)	1	190	100	2
assign	(resid	202	and	name	O4')	(resid	202	and	name	C1')				
	(resid	202	and	name	N9)	(resid	202	and	name	C4)	1	190	100	2
assign	(resid	203	and	name	O4')	(resid	203	and	name	C1')				
	(resid	203	and	name	N9)	(resid	203	and	name	C4)	1	190	100	2
assign	(resid	204	and	name	O4')	(resid	204	and	name	C1')				
	(resid	204	and	name	N1)	(resid	204	and	name	C2)	1	190	100	2
assign	(resid	205	and	name	O4')	(resid	205	and	name	C1')				
	(resid	205	and	name	N1)	(resid	205	and	name	C2)	1	190	100	2

assign	(resid	206	and	name	O4')	(resid	206	and	name	C1')				
	(resid	206	and	name	N9)	(resid	206	and	name	C4)	1	190	100	2
assign	(resid	207	and	name	O4')	(resid	207	and	name	C1')				
	(resid	207	and	name	N9)	(resid	207	and	name	C4)	1	190	100	2
assign	(resid	208	and	name	O4')	(resid	208	and	name	C1')				
	(resid	208	and	name	N9)	(resid	208	and	name	C4)	1	190	100	2
assign	(resid	209	and	name	O4')	(resid	209	and	name	C1')				
	(resid	209	and	name	N9)	(resid	209	and	name	C4)	1	190	100	2
assign	(resid	210	and	name	O4')	(resid	210	and	name	C1')				
	(resid	210	and	name	N1)	(resid	210	and	name	C2)	1	190	100	2
assign	(resid	211	and	name	O4')	(resid	211	and	name	C1')				
	(resid	211	and	name	N9)	(resid	211	and	name	C4)	1	190	100	2
assign	(resid	212	and	name	O4')	(resid	212	and	name	C1')				
	(resid	212	and	name	N1)	(resid	212	and	name	C2)	1	190	100	2
assign	(resid	213	and	name	O4')	(resid	213	and	name	C1')				
	(resid	213	and	name	N1)	(resid	213	and	name	C2)	1	190	100	2
assign	(resid	214	and	name	O4')	(resid	214	and	name	C1')				
	(resid	214	and	name	N9)	(resid	214	and	name	C4)	1	190	100	2
assign	(resid	215	and	name	O4')	(resid	215	and	name	C1')				
	(resid	215	and	name	N1)	(resid	215	and	name	C2)	1	190	100	2
assign	(resid	216	and	name	O4')	(resid	216	and	name	C1')				
	(resid	216	and	name	N9)	(resid	216	and	name	C4)	1	190	100	2
assign	(resid	217	and	name	O4')	(resid	217	and	name	C1')				
	(resid	217	and	name	N9)	(resid	217	and	name	C4)	1	190	100	2
assign	(resid	218	and	name	O4')	(resid	218	and	name	C1')				
	(resid	218	and	name	N9)	(resid	218	and	name	C4)	1	190	100	2
assign	(resid	219	and	name	O4')	(resid	219	and	name	C1')				
	(resid	219	and	name	N9)	(resid	219	and	name	C4)	1	190	100	2
assign	(resid	220	and	name	O4')	(resid	220	and	name	C1')				
	(resid	220	and	name	N1)	(resid	220	and	name	C2)	1	190	100	2
assign	(resid	221	and	name	O4')	(resid	221	and	name	C1')				
	(resid	221	and	name	N9)	(resid	221	and	name	C4)	1	190	100	2
assign	(resid	222	and	name	O4')	(resid	222	and	name	C1')				
	(resid	222	and	name	N1)	(resid	222	and	name	C2)	1	190	100	2
assign	(resid	223	and	name	O4')	(resid	223	and	name	C1')				
	(resid	223	and	name	N9)	(resid	223	and	name	C4)	1	190	100	2
assign	(resid	224	and	name	O4')	(resid	224	and	name	C1')				
	(resid	224	and	name	N9)	(resid	224	and	name	C4)	1	20	100	2
assign	(resid	225	and	name	O4')	(resid	225	and	name	C1')				
	(resid	225	and	name	N9)	(resid	225	and	name	C4)	1	190	100	2
assign	(resid	226	and	name	O4')	(resid	226	and	name	C1')				
	(resid	226	and	name	N9)	(resid	226	and	name	C4)	1	190	100	2
assign	(resid	227	and	name	O4')	(resid	227	and	name	C1')				
	(resid	227	and	name	N9)	(resid	227	and	name	C4)	1	190	100	2
assign	(resid	228	and	name	O4')	(resid	228	and	name	C1')				
	(resid	228	and	name	N1)	(resid	228	and	name	C2)	1	190	100	2
assign	(resid	229	and	name	O4')	(resid	229	and	name	C1')				
	(resid	229	and	name	N9)	(resid	229	and	name	C4)	1	190	100	2
assign	(resid	230	and	name	O4')	(resid	230	and	name	C1')				
	(resid	230	and	name	N9)	(resid	230	and	name	C4)	1	190	100	2
assign	(resid	231	and	name	O4')	(resid	231	and	name	C1')				
	(resid	231	and	name	N1)	(resid	231	and	name	C2)	1	190	100	2
assign	(resid	232	and	name	O4')	(resid	232	and	name	C1')				
	(resid	232	and	name	N1)	(resid	232	and	name	C2)	1	190	100	2
assign	(resid	233	and	name	O4')	(resid	233	and	name	C1')				
	(resid	233	and	name	N1)	(resid	233	and	name	C2)	1	190	100	2

!C3'-endo constraints delta and nu2 only

assign	(resid	202	and	name	C5')	(resid	202	and	name	C4')				
	(resid	202	and	name	C3')	(resid	202	and	name	O3')	1	82	30	2
assign	(resid	202	and	name	C1')	(resid	202	and	name	C2')				
	(resid	202	and	name	C3')	(resid	202	and	name	C4')	1	38	30	2
assign	(resid	203	and	name	C5')	(resid	203	and	name	C4')				
	(resid	203	and	name	C3')	(resid	203	and	name	O3')	1	82	30	2
assign	(resid	203	and	name	C1')	(resid	203	and	name	C2')				
	(resid	203	and	name	C3')	(resid	203	and	name	C4')	1	38	30	2
assign	(resid	204	and	name	C5')	(resid	204	and	name	C4')				
	(resid	204	and	name	C3')	(resid	204	and	name	O3')	1	82	30	2
assign	(resid	204	and	name	C1')	(resid	204	and	name	C2')				
	(resid	204	and	name	C3')	(resid	204	and	name	C4')	1	38	30	2
assign	(resid	210	and	name	C5')	(resid	210	and	name	C4')				
	(resid	210	and	name	C3')	(resid	210	and	name	O3')	1	82	30	2
assign	(resid	210	and	name	C1')	(resid	210	and	name	C2')				
	(resid	210	and	name	C3')	(resid	210	and	name	C4')	1	38	30	2
assign	(resid	211	and	name	C5')	(resid	211	and	name	C4')				
	(resid	211	and	name	C3')	(resid	211	and	name	O3')	1	82	30	2

assign	(resid	211	and	name	C1')	(resid	211	and	name	C2')				
	(resid	211	and	name	C3')	(resid	211	and	name	C4')	1	38	30	2
assign	(resid	212	and	name	C5')	(resid	212	and	name	C4')				
	(resid	212	and	name	C3')	(resid	212	and	name	O3')	1	82	30	2
assign	(resid	212	and	name	C1')	(resid	212	and	name	C2')				
	(resid	212	and	name	C3')	(resid	212	and	name	C4')	1	38	30	2
assign	(resid	213	and	name	C5')	(resid	213	and	name	C4')				
	(resid	213	and	name	C3')	(resid	213	and	name	O3')	1	82	30	2
assign	(resid	213	and	name	C1')	(resid	213	and	name	C2')				
	(resid	213	and	name	C3')	(resid	213	and	name	C4')	1	38	30	2
assign	(resid	214	and	name	C5')	(resid	214	and	name	C4')				
	(resid	214	and	name	C3')	(resid	214	and	name	O3')	1	82	30	2
assign	(resid	214	and	name	C1')	(resid	214	and	name	C2')				
	(resid	214	and	name	C3')	(resid	214	and	name	C4')	1	38	30	2
assign	(resid	217	and	name	C5')	(resid	217	and	name	C4')				
	(resid	217	and	name	C3')	(resid	217	and	name	O3')	1	82	30	2
assign	(resid	217	and	name	C1')	(resid	217	and	name	C2')				
	(resid	217	and	name	C3')	(resid	217	and	name	C4')	1	38	30	2
assign	(resid	218	and	name	C5')	(resid	218	and	name	C4')				
	(resid	218	and	name	C3')	(resid	218	and	name	O3')	1	82	30	2
assign	(resid	218	and	name	C1')	(resid	218	and	name	C2')				
	(resid	218	and	name	C3')	(resid	218	and	name	C4')	1	38	30	2
assign	(resid	219	and	name	C5')	(resid	219	and	name	C4')				
	(resid	219	and	name	C3')	(resid	219	and	name	O3')	1	82	30	2
assign	(resid	219	and	name	C1')	(resid	219	and	name	C2')				
	(resid	219	and	name	C3')	(resid	219	and	name	C4')	1	38	30	2
assign	(resid	220	and	name	C5')	(resid	220	and	name	C4')				
	(resid	220	and	name	C3')	(resid	220	and	name	O3')	1	82	30	2
assign	(resid	220	and	name	C1')	(resid	220	and	name	C2')				
	(resid	220	and	name	C3')	(resid	220	and	name	C4')	1	38	30	2
assign	(resid	221	and	name	C5')	(resid	221	and	name	C4')				
	(resid	221	and	name	C3')	(resid	221	and	name	O3')	1	82	30	2
assign	(resid	221	and	name	C1')	(resid	221	and	name	C2')				
	(resid	221	and	name	C3')	(resid	221	and	name	C4')	1	38	30	2
assign	(resid	222	and	name	C5')	(resid	222	and	name	C4')				
	(resid	222	and	name	C3')	(resid	222	and	name	O3')	1	82	30	2
assign	(resid	222	and	name	C1')	(resid	222	and	name	C2')				
	(resid	222	and	name	C3')	(resid	222	and	name	C4')	1	38	30	2
assign	(resid	229	and	name	C5')	(resid	229	and	name	C4')				
	(resid	229	and	name	C3')	(resid	229	and	name	O3')	1	82	30	2
assign	(resid	229	and	name	C1')	(resid	229	and	name	C2')				
	(resid	229	and	name	C3')	(resid	229	and	name	C4')	1	38	30	2
assign	(resid	230	and	name	C5')	(resid	230	and	name	C4')				
	(resid	230	and	name	C3')	(resid	230	and	name	O3')	1	82	30	2
assign	(resid	230	and	name	C1')	(resid	230	and	name	C2')				
	(resid	230	and	name	C3')	(resid	230	and	name	C4')	1	38	30	2
assign	(resid	231	and	name	C5')	(resid	231	and	name	C4')				
	(resid	231	and	name	C3')	(resid	231	and	name	O3')	1	82	30	2
assign	(resid	231	and	name	C1')	(resid	231	and	name	C2')				
	(resid	231	and	name	C3')	(resid	231	and	name	C4')	1	38	30	2
assign	(resid	232	and	name	C5')	(resid	232	and	name	C4')				
	(resid	232	and	name	C3')	(resid	232	and	name	O3')	1	82	30	2
assign	(resid	232	and	name	C1')	(resid	232	and	name	C2')				
	(resid	232	and	name	C3')	(resid	232	and	name	C4')	1	38	30	2
assign	(resid	233	and	name	C5')	(resid	233	and	name	C4')				
	(resid	233	and	name	C3')	(resid	233	and	name	O3')	1	82	30	2
assign	(resid	233	and	name	C1')	(resid	233	and	name	C2')				
	(resid	233	and	name	C3')	(resid	233	and	name	C4')	1	38	30	2

!C2' -endo constraints delta only

assign	(resid	201	and	name	C5')	(resid	201	and	name	C4')				
	(resid	201	and	name	C3')	(resid	201	and	name	O3')	1	140	50	2
assign	(resid	207	and	name	C5')	(resid	207	and	name	C4')				
	(resid	207	and	name	C3')	(resid	207	and	name	O3')	1	140	30	2
assign	(resid	215	and	name	C5')	(resid	215	and	name	C4')				
	(resid	215	and	name	C3')	(resid	215	and	name	O3')	1	140	50	2
assign	(resid	224	and	name	C5')	(resid	224	and	name	C4')				
	(resid	224	and	name	C3')	(resid	224	and	name	O3')	1	140	50	2
assign	(resid	225	and	name	C5')	(resid	225	and	name	C4')				
	(resid	225	and	name	C3')	(resid	225	and	name	O3')	1	140	50	2
assign	(resid	226	and	name	C5')	(resid	226	and	name	C4')				
	(resid	226	and	name	C3')	(resid	226	and	name	O3')	1	140	50	2

set echo = on end
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