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The synthesis, characterization and refolding of Aminotyrosyl derivatives of Ribonuclease A : a spectroscopic probe for monitoring the CIS/TRANS isomerization of Proline-114

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THE SYNTHESIS, CHARACTERIZATION AND REFOLDING OF AMINOTYROSYL DERIVATIVES OF RIBONUCLEASE A: A SPECTROSCOPIC PROBE FOR MONITORING THE CIS/TRANS ISOMERIZATION OF PROLINE-114.

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ABSTRACT

THE SYNTHESIS, CHARACTERIZATION AND REFOLDING OF AMINOTYROSYL DERIVATIVES OF RIBONUCLEASE A: A SPECTROSCOPIC PROBE FOR MONITORING THE CIS/TRANS ISOMERIZATION OF PROLINE-114

by Bhagya Puntambekar

Chemical modification of specific amino acid residues with spectroscopic probes provides a means by which folding about discrete regions of a protein can be monitored. The thrust of this investigation centers around monitoring the cis/trans isomerization of Pro-114 in ribonuclease A via aminated derivatives of the neighboring Tyr-115.

We have synthesized, purified to homogeneity, and characterized derivatives of ribonuclease A in which Tyr-115 and both Tyr-115 and Tyr-76 have been nitrated and aminated. The refolding kinetics were monitored at 10°C (pH 5.5) and -15°C (pH 6, 35% methanol). Triphasic kinetics were observed under all conditions. The rate constants observed for the slowest phase are consistent with that expected for proline isomerization. The amplitudes associated with the slowest phase represent 50% of the total expected when urea was used to unfold the protein and 57% for the thermally unfolded protein and thus corroborate the proton NMR results by Biringer (unpublished).

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LIST OF ABBREVIATIONS

1. APP: Aminopeptidase
2. 2′:3′ CMP: 2′:3′ Cyclic monophosphate
3. $C^{\varepsilon 115}$: Modification of tyrosine-115 at the epsilon position
4. $C^{\varepsilon 115,76}$: Modification of tyrosine-115 and tyrosine-76 at the epsilon position.
5. CD : Circular Dichroism
6. HEPES: N-2-Hydroxyethylpiperazine-N′-2-ethane Sulfonic acid.($C_8H_{18}N_2O_4S$)
7. HPLC : High pressure liquid chromatography.
8. IEF: Isoelectric Focussing
9. I_N :Native-like intermediate
10. IR : Infra-Red Spectrum.
11. ISP: Isomer specific proteolysis
12. N: Native state
13. NMR: Nuclear magnetic resonance.
14. ORD: Optical rotatory dispersion
15. RNase A: Bovine pancreatic ribonuclease A.
16. TNM: Tetranitromethane
17. U: Unfolded state.

INTRODUCTION

1.1. PROTEIN FOLDING PROBLEM

The study of protein chemistry centers around the assembly of polypeptide chains from free amino acids, folding of these chains into native three-dimensional structures and the relationship between their detailed geometry in solution and their functions. The creation of a protein in a living cell begins with the biosynthesis of polypeptide chains. To be biologically active, all proteins must adopt highly specific three-dimensional structures. The genetic information for a protein specifies only the primary structure, the linear amino acid sequence, which forms the polypeptide backbone. These polypeptides interact with their aqueous or lipid environments to fold up into discrete, highly organized and tightly packed three-dimensional conformations. The fact that a particular three-dimensional conformation depends on the primary sequence indicates that the three-dimensional structure is determined by primary sequence (1). The precision and reproducibility of this folding is so high that polypeptide chains with the same amino acid sequences always arrive at homogeneous conformations (3),(4).

The detailed mechanism by which the primary sequence dictates for the secondary and tertiary structures is not

fully understood. This came to be known as "the protein folding problem" and has been a major challenge in biophysical chemistry for the last 20 years (2).

Each protein has a unique, precisely defined amino acid sequence which is genetically determined. The sequence of nucleotides in DNA specifies a complementary sequence of nucleotides in RNA, which in turn specifies the amino acid sequence. It is this sequence which "codes" for the formation of secondary and tertiary structure found in a protein's native structure. Hence, knowledge of the "code" would allow one to deduce the native structure from the amino acid sequence which in turn is important for the understanding of the molecular basis of its biological activity. Knowledge of the "code" would aid in the engineering of protein structures using recombinant DNA techniques and provide a tool to specify hypothetical functions for given amino acid sequences (5),(6),(7). Moreover, alterations in amino acid sequence can result in an abnormal function and disease. Therefore, sequence, which is responsible for a protein's native structure and biological function is also a part of molecular pathology and pharmacology.

1.2. THE PROPERTIES OF THE STABLE CONFORMATIONAL STATES OF PROTEINS

1.2.1.THE NATIVE FOLDED STATE (N)

Protein conformations differ in the relative orientations of the amino acid side chains and in the angle of rotation about the bonds of the backbone. Most proteins in nature are oligomeric. Interdomain and subunit interactions further complicate the problem. The spatial arrangement and subunit interactions define the specificity of the three-dimensional structure of proteins (7).

The proteins with non-homologous amino acid sequences usually have very different conformations. However, proteins with homologous sequences invariably have similar conformations, the conspicuous characteristic being the conservation of the non-polar character of the side chains at specific sites along the polypeptide chain.

Folded proteins are not static structures and they exhibit various degrees of flexibility, the greatest at the surface and least in the interior. This has a direct relevance to protein folding where it reflects the free energy constraints on unfolding and refolding. Small changes in pH, temperature and solvent can lead to changes in the net stability of protein molecules (50). The folded conformation of a small globular protein or a domain in a large protein is apparently in a relatively narrow free energy minimum and any

perturbation of that folded conformation requires significant increase in free energy. These free energy changes are frequently on the order of 40KJ/mol.

A protein molecule is a macroscopic system consisting of thousands of atoms which participate in thermal motion. Therefore, understanding a protein requires the knowledge of its thermodynamics. Information on the energetic basis of a protein structure can be obtained by using temperature as a variable because temperature and energy or enthalpy of a system are conjugate intensive and extensive variables that determine the state of a macroscopic system. The thermodynamics of unfolding transitions have been characterized calorimetrically for several proteins. For example, unfolding transitions of plasminogen were studied over a broad temperature range by Privalov (11),(12),(13). The thermodynamic analysis of the excess heat capacity revealed that the temperature induced disruption of the native structure of plasminogen was a process consisting of seven transitions and each of these transitions proceeds with the absorbance of a considerable and definite amount of energy. It has also been observed that the enthalpies (ΔH) and entropies (ΔS) are temperature dependent. The heat capacity of the unfolded state is significantly greater than that of the folded state. Privalov explains this difference in heat capacity as a result of the temperature dependent ordering of water molecules around the hydrophobic portions of the protein which are solvent accessible when a protein

unfolds.

Hydrophobic interactions are a major contributor to the stability of the folded state. However, there may be other factors that stabilize the protein molecules (9),(10). Interactions within the folded state can have substantially lower free energies than those between the solvent and the exposed portions of the protein. Most hydrogen bonds within water and between protein and water are usually present for a fraction of time, but those present within folded proteins are present most of the time and will have a larger negative enthalpy. Van der Waal's interactions within the protein interior should be substantially greater than those between the protein and the solvent and hence possess lower enthalpies. Therefore, most of the interactions within the folded protein are energetically more favorable, both in enthalpy and free energy than the corresponding interactions of the unfolded state.

1.2.2. THE UNFOLDED STATE (U)

"The distinctive transition associated with the loss of the highly folded state of proteins is termed as denaturation or unfolding" (4).

The model unfolded protein consists of an ensemble of random structures, all conformations having comparable free energies. However, evidence exists that unfolded proteins are not true random coils even under extreme conditions of pH,

temperature or in the presence of denaturants (50),(51). Their conformation is dependent on the energetics of interactions between different parts of a polypeptide fragment in a random coil and the solvent. If the interactions between different parts of a polypeptide chain are favorable over the solvent, a structure more compact than a random coil results. On the contrary, if the interactions are energetically unfavorable, a random coil would form (1). Unfolded states produced under different unfolding conditions have different properties and they are distinguishable thermodynamically.

1.3. KINETIC STUDIES OF PROTEIN UNFOLDING AND REFOLDING

Proteins refold quickly as they follow a defined pathway and do not rely on random fluctuations. Elucidating the mechanism of protein folding requires characterization of the initial, final and intermediate conformational states and the steps by which they are interconverted (1),(3),(7),(8).

In the unfolded state, every molecule of a typical population is likely to have a unique conformation at each instant of time. The question that arises is whether this heterogeneity is apparent in the refolding kinetics, that is, whether the refolding rates for each conformation can be determined and whether all the molecules follow a common folding mechanism. Protein folding is a complex event and not all the details can be elucidated. It may be feasible to

determine the common pathway, characterize the slowest transitions, conformations and energetics of the most stable intermediates.

1.3.1. PROTEIN FOLDING STUDIES ON RIBONUCLEASE A

Ribonuclease A is a small globular protein, an enzyme that hydrolyzes ribonucleic acid. It consists of a single polypeptide chain of 124 amino acid residues and four disulfide linkages, four prolines (Pro) and six tyrosine (Tyr) residues. The x-ray structure of RNase A shows that three tyrosines are solvent exposed (Tyr-115, -76 and -73) and three are buried in the hydrophobic core (Tyr-25, -92 and -97). The peptide bond involving Pro-114 and Pro-93 are cis in the native conformation and isomerize to the trans form in the equilibrium unfolded species.

It was in 1964 that Anfinsen performed the first folding experiments on RNase A. When RNase was treated with β-mercaptoethanol in 8 M urea, a fully reduced, randomly coiled polypeptide chain devoid of enzymatic activity was produced. Anfinsen observed that when RNase was freed of urea and β-mercaptoethanol, it gradually regained the enzymatic activity. The sulfhydryls of the denatured enzyme became oxidized by air and the enzyme spontaneously refolded into a catalytically active form under suitable refolding conditions. Based on the results of these experiments,

Anfinsen made a critical observation. He postulated that the information needed to specify the complex three-dimensional structure of ribonuclease is contained in its amino acid sequence and that the native structure of a protein is thermodynamically the most stable form (14).

Since then, the folding of RNase A has been examined in several laboratories. A major breakthrough came in 1973, when Garel and Baldwin (15) discovered that there were both fast and slow folding species of RNase. In their experiments, the relative exposure of all the six tyrosines was monitored by absorbance at 286 nm. They observed a biphasic reaction in which there was a 100-fold difference in the rates. Although they folded with different rates, both the fast and slow refolding species possessed the same molar absorbancy at 286 nm and failed to bind the competitive inhibitor, 2',3' cyclic monophosphate (15). Based on their data and the data observed by others, a model was proposed for the refolding mechanism (15),(16),(17),(18),(19):

$$U_1(U_s) \text{ --- slow ---> } U_2(U_f) \text{ --- fast ---> } N$$

where in the early studies U_1 and U_2 referred to the slow folding and fast folding species. These were eventually changed to U_s and U_f to emphasize their occurrence along the folding pathway.

One of the explanations suggested for the difference in folding rates was the slow cis-trans isomerization of the

proline residues. In 1975, Brandts et al.(18) studied the involvement of proline residues in model compounds. The activation energies were examined for the refolding reactions at 25°C. They observed that the kinetic phase associated with the isomerization of proline residue was the slowest process and had a high activation energy. These results supported the idea that proline isomerism is involved in the slow process observed in protein folding reactions. Inspection of the space filling models also revealed that the isomerism is sterically influenced by the bulkiness of side chains which are adjacent to prolines in polypeptide chains (18). Imide bonds, like peptide bonds are planar and have partial double bond character and thus possess a high activation enthalpy for the cis-to-trans isomerization

The second explanation suggested by Nall et al. in 1978 for the slow folding species was the involvement of the loop-threading reactions involving the "loops" formed by the disulfide bonds (20). They suggested that RNase has three major overlapping disulfide bonded loops: A (26-84), B (40-95) and C (58-110) and two arrangements of these loops are possible (1 &11) (Figure 1).

One could either pull the C-terminal or the N-terminal end through B which involves pulling one entire loop through the other. They suggested that such a loop-threading reaction is certain to be slow. Whether the isomerization occurs after unfolding to give the major fraction of arrangement 11 (Figure 1) was not known. However, if this occurs, an

increase in entropy would be observed. But if $U_1(U_s)$ ----> $U_2(U_f)$ reaction involves the interconversion of structure 11 to 1 (Figure 1), a small activation enthalpy would be expected (20).

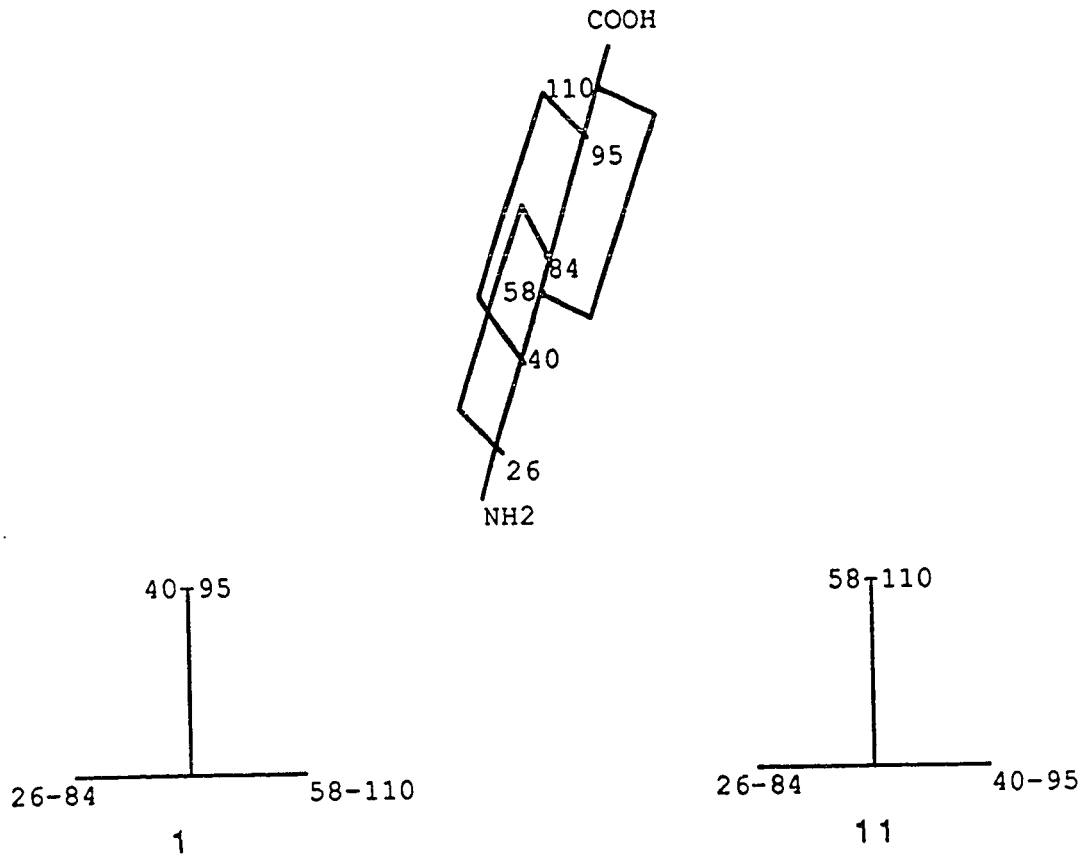

FIGURE 1: Two conformational isomers of the polypeptide chain of RNase involving the three overlapping disulfide bonded loops. The interconversion of these two isomers may be possible by pulling or poking one loop through another and may thus be a slow reaction.

It was proposed that if $U_1(U_S)$ is produced by the cis-trans isomerization of proline residues about X-Pro imide bonds, then the formation of U_1 should be catalyzed by strong acids (21). Acid catalysis experiments were performed by Baldwin et al.(23). After rapid unfolding of the native protein in concentrated $HCLO_4$ at 0°C, the rate of slow formation of U_1 was measured. Catalysis of its formation was found at different $HCLO_4$ concentrations above 5 M. The acid catalysis of the formation of U_1 and its high activation enthalpy were consistent with the rate limiting step being the isomerization of an X-proline bond as suggested by Brandts (18). The existence of two acid catalyzed reactions that were distinguishable by the $HClO_4$ concentrations suggested that at least two essential proline residues were involved in producing the slow folding species (23). U_f has all the essential prolines in the same configuration as the native enzyme (N) and thus refolds rapidly (U_f ---> N, τ = 50 ms at pH 6, 25°C), whereas U_S has at least one incorrect proline isomer and therefore folds more slowly.

The x-ray structure of RNase shows that Pro-114 and Pro-93 are cis in the native protein. In 1983, Lin and Brandts performed experiments to study the involvement of Pro-93 in the refolding reaction. They observed that trypsin cleaves the Lys-X-Pro bonds in a peptide chain when the proline following the active bond was in the trans isomeric state. However, the cis form must isomerize to trans form before it

is cleaved. Taking advantage of this isomer specific proteolysis, the sequence -Lys_{91}-Tyr_{92}-Pro_{93}- was examined in oxidized RNase A. They observed that Tyr-Pro bond exists 33% in the cis form at equilibrium and the cis-to-trans relaxation time for isomerization is 5.0 minutes at 10°C (40). Since then Lin and Brandts performed several experiments to study the unfolding and refolding of RNase A (37),(38),(39),(40). All the results have shown that Pro-93 is 100% cis in the native protein and 70% cis in the reversibly unfolded protein.

In 1978, Nall et al. (20) found that the refolding reaction U_S ---> N shows a small apparent activation enthalpy at low concentrations of guanidine hydrochloride. They interpreted this in terms of alternate folding pathways of U_S which involve structural intermediates prior to proline isomerization. This was later proven by NMR studies (24).

Presently, three different pathways of refolding have been detected which originate from distinct unfolded states and fold to native RNase in parallel reactions (25),(26); the numbers in the parentheses refer to the percent of molecules involved in the three processes.

U_f ---fast---> N (20%)

Us^{11} ---slow---> N (60-70%)

Us^{1} ---very slow---> N (10-20%).

Examination of the Us^{11} to native state in the folding pathway revealed the presence of two intermediate structures. A hydrogen bonded intermediate (I_1) was identified by measuring the competition between the amide proton exchange and refolding (20),(27),(28). A compact intermediate (I_N) which accumulated late in the folding process was also observed. I_N was shown to have many properties similar to the native protein. It had a folded globular structure providing a hydrophobic environment for part of the tyrosine residues as in the native RNase and a binding site for the specific inhibitor 2′ CMP. However, the fluorescence properties differed, suggesting that at least one proline was in an incorrect configuration (26),(29). It was shown that I_1 and I_N can be populated under strongly native folding conditions and it was also suggested that Pro-93 was in an incorrect trans configuration in I_N. The folding pathway proposed was:

$$U_S^{11} \text{----->} I_1 \text{----->} I_N \text{----->} N$$

Refolding kinetics of RNase at subzero temperatures were studied by Biringer and Fink (30),(31),(32) by monitoring the intrinsic fluorescence and absorbance signals from the six tyrosine residues and specifically modified tyrosine residues, inhibitor binding and catalytic activity. Multiphasic kinetics were observed.

A model was proposed for the slow pathway:

U_S^{11} ---> I_1 ---> I_2 ---> N (50%)

U_S^{1} ---> I_1 ---> I_2 ---> I_3 ---> N (30%)

The simplest system consistent with the results for the slow refolding species involved two parallel pathways with multiple intermediates on each pathway. It was observed that 30% of the unfolded state refolds by minor pathway (Us^1) which involved the isomerization of Pro-93. The major pathway (Us^{11}) involved 50% of the unfolded state; the reason for its slow refolding was not apparent. However, a native-like intermediate formed considerably more rapidly in the major slow refolding pathway as compared to the minor pathway (31),(32).

In 1988, Lin and Brandts conducted experiments to determine the structural properties of the intermediate structure (I_N). Folding was initiated and samples were applied to rapid HPLC gel filtration at various times during the folding reaction. This allowed for the separation of I_N. Upon comparison of the folding kinetics, they found that the change from I_N to N was associated with a small change in fluorescence and thus I_N is not associated with proline isomerization. The fluorescence assay for isomerization of Pro-93 showed that I_N exists with Pro-93 in the cis state, identical to native RNase. It was concluded that I_N was not

associated with Pro-93 as suggested earlier (26),(29).

1.3.2. REFOLDING STUDIES ON RNase MONITORED BY NITROTYROSINE ABSORBANCE

One approach useful in elucidating the refolding reaction of proteins is to identify steps in the folding process of a particular region in a polypeptide chain. However, placement of different spectroscopic probes into specific regions of the structure allows one to monitor structural changes in particular regions of the proteins independently. Chemical modification of existing amino acid side chains has proven to be a useful method to introduce such spectroscopic probes into proteins. Although there are a number of intrinsic spectroscopic probes within the molecule itself, time dependent changes in their properties provide only a global picture of the folding process.

One of the modifying reagents to be completely specific in its reactivity is tetranitromethane (TNM). This reagent introduces a 3-nitro group in tyrosyl residues. This results in the formation of a new absorption band and a large change in the pK of the phenolic hydrogen of tyrosine (33),(34),(35).

The nitrotyrosine has a pK near neutrality. The absorption band in the visible region changes markedly with the degree of ionization and thus the pK changes can be monitored spectrophotometrically. Garel and Baldwin (36)

exploited this phenomenon to follow the refolding kinetics of nitrated RNase. A trinitrated derivative ($C^{\varepsilon 115,76,73-NO_2}$) of RNase was prepared for this purpose.

The reported refolding kinetics were biphasic. The faster phase resembled the one observed in the refolding kinetics of unmodified RNase in terms of the rate, activation enthalpy and dependence on guanidine hydrochloride concentration. For the slower phase, the rate and activation enthalpy were independent of guanidine hydrochloride concentration. Since such independence had been associated with proline isomerization, it was concluded that the slow phase was limited by this process (36).

The kinetics of refolding of nitrotyrosine derivatives was also studied by Biringer and Fink (41). Derivatives of ribonuclease A in which Tyr-115, Tyr-115,76 and Tyr-115,76,73 were nitrated and purified to homogeneity. The refolding kinetics for each derivative were monitored by absorbance at -15°C, pH 3.0 and 6.0 by using a cryosolvent composed of 35% aqueous methanol.

At both the pH values, biphasic kinetics were observed for Tyr-115. The data indicated that a significant portion of the protein (63% at pH 3 and 74% at pH 6) attained a native like environment about Tyr-115 within 16 sec. The remainder becomes native within 600 sec at pH 3 and 200 sec at pH 6.0. The Tyr-115,76 derivative showed triphasic kinetics at both pH values. The amplitudes indicated that 53% of the native

signal for Tyr-76 returned in the fastest phase at pH 3 and 75% at pH 6.0.

The results indicated that different regions of the molecule refold at different rates, implying a sequential pathway in which partially folded intermediates exist. The similarity in the rates for both the modified and unmodified RNase suggested that the pathway of folding is not significantly perturbed by nitration of these tyrosines. Although the nitrated Tyr-115 was expected to reveal the changes in the local environment, no slow phase corresponding to the isomerization of Pro-114 was observed (41).

1.4. PROLINE ISOMERIZATION

Conformational heterogeneity of the unfolded state has been shown to be due to the presence of a mixture of slow and fast refolding molecules. This heterogeneity results at least in part from the isomerization state of peptide bonds preceding proline residues (42).

Peptide bonds formed by amino acid residues other than proline have a thermodynamic preference for the planar trans configuration. This planarity is due to the double bond character of the peptide linkage. The trans form is preferred due to more favorable interactions and a large conformational entropy relative to the cis form. In contrast, the peptide bond formed between a proline residue and the immediately preceding amino acid residue is more likely to exist as a

mixture of cis and trans form in solution because of the added steric constraints associated with the linkage of proline side chain to the peptide nitrogen atom (1),(42). As noted previously, the process of proline isomerization is characterized by slow kinetics and a high energy barrier.

Protein conformational stability and proline isomerization are linked functions; whatever effect the protein conformation has on the stability of the cis and trans isomers of a peptide bond, the isomer must have exactly the same effect on the stability of the folded conformation. When a protein is unfolded, the constraints favoring one form over the other are removed and an equilibrium between cis and trans forms is attained. When a protein refolds, a fraction of molecules (U_f) will have all the necessary peptide bonds in the correct isomerization state and therefore is responsible for fast refolding. Others (U_s) will have one or more prolines in the incorrect isomerization state (1),(42).

For large polypeptides and proteins, the role of cis-trans isomerization in most cases has been inferred indirectly by comparing thermodynamic and kinetic parameters with those of model peptides. The properties of proline isomerization are known to be greatly influenced by various parameters such as amino acid sequence, ionization, chain length and chain dynamics and disulfide bond linkage. Therefore, the isomerization of proline residues in proteins can be very different from those in model peptides. It is difficult to ascertain the involvement of proline

isomerization in the kinetic phases, structural intermediates or the phases in which both the processes are involved (1),(42). The inability to obtain direct information on proline isomerization leads to difficulty when interpreting the protein folding data.

1.4.1. PROLINE ISOMERIZATION AND SLOW FOLDING SPECIES IN RNase

A quantitative study of the unfolding and refolding kinetics of RNase A reveals a three species mechanism (15),(18)

$$N \text{ ----> } U_f \text{ ----> } U_s.$$

The fast folding species comprises only 20% of the unfolded molecules and is not affected by high temperatures or strong denaturants such as 6 M guanidine hydrochloride or 8.5 M urea (15),(22). Kinetics of the U_f to U_s reaction in the unfolded protein were compared to the cis-trans isomerization of proline residues in model compounds. The results provide strong evidence that U_f to U_s involves proline isomerization. The striking characteristic observed for both the reactions were: high activation enthalpy (≈20 Kcal/mol), catalysis by strong acids and kinetics that are independent of guanidine hydrochloride (23).

1.4.2. UNFOLDING ASSAY

A slow refolding double jump assay was developed by Schmid (52) to probe the sequential nature of the N to U_f unfolding step and the subsequent U_f to U_s reaction. The amount of U_s formed after different times of unfolding can be determined by this slow refolding assay. Samples are withdrawn at regular time intervals after the initiation of unfolding and transferred to standard refolding conditions. The amplitude of the slow folding reaction is then determined. The amplitude of the slow reaction is proportional to the concentration of the slow folding species which is present at the time when the sample is withdrawn for the assay. The amplitudes increase with the time of unfolding and thus yield the kinetics of the formation of the slow folding species. These kinetics could be fitted to a single exponential curve and the rate constants determined for the slow process. It was suggested that since high activation enthalpy is associated with proline isomerization, the reaction can be strongly decelerated at low temperatures.

It was predicted that if the unfolding (N to U_f) and then the slow isomerization (U_f to U_s) are similar in rate at equilibrium, the slow phase would show an initial lag phase. However, if the initial unfolding reaction is rapid, there is no coupling between folding and isomerization, and the double jump assays can be fitted by a single exponential. In addition, a coupling between unfolding and the isomerization

leads to sigmoidal kinetics.

1.4.3. ISOMER SPECIFIC PROTEOLYSIS:(ISP)

RNase has four prolines of which Pro-117 and Pro-42 appear to be trans in the native structure as observed by X-ray crystallography. However, Pro-114 and Pro-93 are cis in the native state and isomerize to a mixture of cis and trans isomers in the equilibrium unfolded species. Refolding studies on RNase have confirmed the presence of both fast and slow folding species and that the slow phase involved proline isomerization. It has been shown indirectly that Pro-93 is involved in at least one of the folding pathways (37),(40).

It is difficult to experimentally monitor the proline isomerization process directly. Lin and Brandts reported a unique methodology to study the isomerization process. It was established in 1979 that "prolidase," an exodipeptidase that hydrolyzes only X-Pro dipeptides (X = any amino acid except proline), and aminopeptidase P (APP), a proline specific exopeptidase that hydrolyzes the N-terminal X-Pro bond, exhibit absolute specificity towards the trans form of the X-Pro bond (43),(44),(45). Later, it was shown that endopeptidases such as trypsin and proline-specific endopeptidases (40),(54) also exhibit specificity towards X-Pro bond. It was found that trypsin can cleave Lys-X-Pro bond in a substrate when X-Pro is trans while proline specific endopeptidase can cleave Pro-Y bond in X-Pro-Y, when X-Pro

bond is in a trans configuration. Lin and Brandts took advantage of this isomeric specificity to study the thermodynamic and kinetic properties of isomerization for particular proline residues in peptides.

Isomer Specific Proteolysis experiments are initiated by mixing a substrate solution (the protein solution to be examined) with an isomer specific endopeptidase. The protease then cleaves the particular peptide bond in which the adjacent prolines are in the correct isomerization state. Aliquots of the reacting solution are then pipetted into inhibitor solution to stop the protease activity. If the hydrolysis products cannot be measured directly, they are further hydrolyzed with an exopeptidase to release a free amino acid. The amount of amino acid released is thus a measure of the number of protein molecules with the particular proline in the correct isomerization state. The protein substrates in which the active bonds are not accessible to rapid hydrolysis must be irreversibly unfolded first before the hydrolysis is initiated.

At high enzymic activity, isomerization is slow relative to the rate of hydrolysis. If, under these conditions, hydrolysis is monitored as a function of time, information regarding the thermodynamic and kinetic properties of proline isomerization can be obtained. Two kinetic phases will be seen if the X-Pro bond of the substrate exists as a mixture of cis and trans forms. A fast phase, whose rate of hydrolysis strongly depends on enzyme concentration and

corresponds to the hydrolysis of the trans form. However, the relaxation time of the slower phase is independent of enzyme concentration and corresponds to the cis-to-trans isomerization and subsequent hydrolysis. A semilog plot of substrate concentration vs. time shows the fast and slow phases as first order processes. The relative amplitude of the fast to slow phase is equal to the trans/cis ratio existing for the peptide in the initial state. The relaxation time of the slow phase is equal to the inverse rate constant for the cis-trans isomerization in solution (42).

Lin and Brandts (42) used this ISP method to study the isomerization of Pro-93 in RNase. They attempted to use trypsin in tandem with aminopeptidase P and study the isomerization of Tyr-Pro bond in the sequence Lys_{91}-Tyr_{92}-Pro_{93}-. RNase was first irreversibly denatured by a short pepsin pulse and then incubated in trypsin solution. The fragment 92-98 was further hydrolyzed by high APP activity to release Tyr_{92}. The release of free tyrosine as a function of incubation time with trypsin was plotted. The slow kinetic phase was attributed to the cis to trans isomerization state of the Tyr_{92}-Pro_{93} bond and the subsequent hydrolysis of the Lys_{91}-Tyr_{92} bond. The fast phase corresponded to the cleavage of the trans form. The total amplitude of the slow phase obtained by extrapolation would then give the percent of cis form present in the native RNase. The results indicated that Pro-93 is 97% cis in the native state, 70% cis in the denatured RNase. The isomerization of the Tyr_{92}-Pro_{93} bond

during the refolding of RNase was also investigated by using the same experimental approach. RNase was refolded for a period of time before the pepsin-trypsin treatment was initiated to determine the cis/trans content at that particular time along the reaction pathway. For each refolding time, six different incubation times with trypsin were examined. The results are in agreement with the data obtained from isomer specific proteolysis.

The involvement of Pro-114 during the folding process was also examined by Lin and Brandts (53) by using the method of isomer specific proteolysis. Chymotrypsin was the protease examined for studying the sequence Gly_{112}- Asn_{113}-Pro_{114}-Try_{115}-Val_{116}-Pro_{117}.

It was observed that chymotrypsin cleaves the Tyr_{115}-Val_{116} bond completely in ≈1 minute for oxidized, native and equilibrium unfolded RNase. Once the Tyr_{115}-Val_{116} bond is cleaved by chymotrypsin, the carboxy-terminal Pro_{114}-Tyr_{115} would be an excellent substrate for proline specific endopeptidase only if Asn_{113}-Pro_{114} bond is trans.

This procedure was followed to study the isomerization of Pro-114. The refolding kinetics were also monitored by absorbance. The results indicated that the isomerization about Pro-114 is involved in the folding of RNase A, but only 5% of the unfolded molecules are involved (53).

1.4.4. NMR STUDIES

The ^{1}H NMR spectra for the partially and fully unfolded nitrotyrosyl derivatives of RNase were studied by Biringer (unpublished data). The proton spectra of native $C^{\varepsilon 115\text{-}NO_2}$ RNase showed a singlet for the δ-proton adjacent to the nitro group as expected. However, the thermally unfolded spectrum showed two resonances for this proton with a total area equivalent to a single proton. This indirectly indicated a slow equilibrium process. Thermally unfolded spectra were examined for $C^{\varepsilon 115,76\text{-}NO_2}$ and $C^{\varepsilon 115,76,73\text{-}NO_2}$ RNases, which also showed this multiplicity. Comparison of the peak areas indicated that the two resonances were produced only by nitrotyrosine-115. The thermally unfolded spectrum for $C^{\varepsilon 92\text{-}NO_2}$ RNase also revealed dual peaks for the δ-proton and the corresponding spectrum for $C^{\varepsilon 97\text{-}NO_2}$ RNase revealed a single resonance. Since only Tyr-115 and Tyr-92 are adjacent to the proline residues, it was suggested that the dual nature of the resonances is due to the isomerization state of the neighboring prolines.

The area ratio of the resonances observed for $C^{\varepsilon 92\text{-}NO_2}$ RNase was 30:70, very close to the cis-trans ratio reported

by Lin and Brandts for Pro-93 in urea unfolded RNase A. The area ratio of the resonances for the δ-proton of thermally unfolded $C^{\varepsilon 115\text{-}NO_2}$ RNase were 45:55. If these results indicate the isomerization state of Pro-114, this result contradicts that observed by Lin and Brandts (95:5 cis-trans ratio, by isomer specific proteolysis).

The NMR spectra of $C^{\varepsilon 115\text{-}NO_2}$ RNase unfolded in 5 M guanidine hydrochloride presented a slightly different ratio for these resonances (62:38) (Biringer, unpublished data). Therefore, a discrepancy does exist in the observed results. The possible explanation for this difference could be the effect of local environment or the denaturant dependence of cis/trans isomers of proline residues. A more sensitive spectroscopic technique such as fluorescence could reveal the information regarding the contribution of Pro-114.

1.5. SPECIFIC AIMS

1.5.1. SYNTHESIS OF AMINOTYROSYL DERIVATIVES OF RNase

The involvement of proline isomerization in the folding of RNase has been the subject of controversy. It has been generally accepted that Pro-93 is involved in at least one of the folding pathways. The isomer specific proteolysis (ISP) method employed by Brandts (42) and the NMR data on nitrated derivatives by Biringer (unpublished data) for Pro-114 have shown contradictory results.

The refolding kinetics for the nitrotyrosines were monitored by absorbance. However, no slow phase corresponding to the isomerization of Pro-114 was observed. Apparently, absorbance proved to be insensitive to the process.

Substitution on the aromatic ring causes shifts in the wavelength of absorption maxima and corresponding changes in the fluorescence peaks. Fluorescence efficiency is also affected. Though tetranitromethane has been shown to be very specific towards tyrosine residues (33),(34),(35), it is not of direct use since nitrotyrosines are not fluorescent.

However, aminotyrosines are highly fluorescent. Earlier studies (46),(47),(48) have shown that nitrated tyrosines can be easily reduced to amino tyrosines by a reaction with dithionite ion. The range of emission for the aminotyrosyl residues of 320-400 nm with the optimal activation of 288 nm permits one to distinguish fluorescence of aminotyrosyl

residues from that of tyrosyl residues (A/E = 276/305 nm). In addition, emission from the aminotyrosyl residues was shown to be very sensitive to the environment. This property would allow an independent examination of modified and unmodified tyrosines in RNase A (46).

The specific aim of this project was to synthesize and purify the aminotyrosyl derivatives of RNase A ($C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase), to study the properties of these derivatives, and to detect and characterize the intermediates along the folding pathway and determine the involvement of Pro-114.

MATERIALS AND METHODS

2.1. MATERIALS

Ribonuclease A (Bovine Pancreas), 3-Amino Tyrosine, Ampholytes (pH 8-10.5), Sephadex G-200 (IEF resin) and Cytidine 2'-3' cyclic monophosphate (98%) were purchased from Sigma Chemical Company; Guanidine Hydrochloride (ultra pure) and Urea (ultra pure) from Schwarz/Mann Biotech; Tetranitromethane from Aldrich Chem. Co., Inc. Dithionite (Sodium Hydrosulfite) was purchased from J.T. Baker Chem. Co.

All the fluorescence experiments were carried on a Perkin-Elmer LS-3 Fluorescence Spectrophotometer and all the absorbance measurements were taken on a Hewlett Packard 8452 Diode Array Spectrophotometer.

2.2. METHODS

2.2.1. CHEMICAL MODIFICATION AND PURIFICATION

2.2.1.1. Preparation of $C^{\varepsilon 115\text{-}NO_2}$ RNase and $C^{\varepsilon 115,76\text{-}NO_2}$ RNase

Nitration of the solvent exposed tyrosine (Tyr-115 and Tyr-76) residues was accomplished by a reaction of RNase with tetranitromethane (TNM). The procedure followed was similar to that of Biringer et al.(41).

In a typical preparation, 50 mg of the protein was dissolved in 20 mL of borate buffer (0.1 M) at pH 8.0. A thirty fold molar excess of TNM was added as a freshly prepared 8% (v/v) solution in 90% (v/v) ethanol. This concentration of TNM produces predominantly singly and doubly nitrated derivatives ($C^{\varepsilon 115\text{-}NO_2}$ RNase and $C^{\varepsilon 115,76\text{-}NO_2}$ RNase)(41). The reaction was allowed to proceed for 20 minutes at room temperature with constant stirring. The reaction mixture was then eluted through a G-25 column (2.5 X 20 cm) with 0.1 M ammonia. The nitrated RNase and nitroformate (a major by-product) were separated by this method. This procedure was repeated for three additional 50 mg samples of protein. The nitrated protein from all the samples was combined, dialysed exhaustively to remove ammonia and any residual nitroformate, and then lyophilized.

The reaction of TNM with proteins proceeds by a free radical mechanism and results in the production of some covalently cross-linked dimers. The dimers were removed chromatographically. The procedure used was as follows: the lyophilized material was dissolved in about 2 mL of 0.15 M phosphate, pH 6.5, loaded on a G-75 column (2.5 X 20 cm), and eluted with the same buffer. The monomeric fraction was dialysed, frozen and lyophilized.

2.2.1.2. Purification of Nitrated Derivatives of RNase

The nitrated RNase produces a mixture of various derivatives and unmodified RNase. The separation of individual pure components was accomplished by preparative iso-electric focussing.

A glass plate (10 X 20 cm) was coated with a layer of 5.6% (w/v) gel suspension containing Sephadex IEF resin and 6.4% (v/v) ampholytes (pH 8-10.5). Wicks, previously soaked in the anode (HEPES, 0.1 M), and cathode (NaOH, 0.1 M) buffers were placed on either ends of the plate and electrodes were positioned. The plate was placed in the electrophoresis chamber precooled with chilled water (0°C). The chamber was covered, and the cell prefocussed for 45 minutes at constant power of 12 watts. The chamber was continuously purged with nitrogen to avoid condensation and absorption of CO_2 by the gel bed.

The lyophilized monomeric sample was dissolved in a minimum of carrier ampholytes (pH 8-10.5). After prefocussing, the sample was applied along the center of the gel bed. The gel was electrophoresed at 32-36 watts until three distinct yellow colored bands appeared on the gel (3-4 hrs.), the first being $C^{\varepsilon 115\text{-}NO_2}$ RNase followed by $C^{\varepsilon 115,76\text{-}NO_2}$ RNase and $C^{\varepsilon 115,76,73\text{-}NO_2}$ RNase. The bands were scooped from the plate and the gel beads were washed in sintered glass filtering funnels to separate the protein from

the resin. The three samples were individually dialysed and lyophilized. The lyophilized protein samples were checked for purity by analytical iso-electric focussing.

2.2.1.3. Preparation of Aminated Derivatives

The nitrated species were reduced to the corresponding aminated derivatives by a reaction with dithionite ion (sodium hydrosulfite). A typical preparation involved the reaction of each of the nitrated derivatives (10 mg/mL; 0.15 mM) with 75 fold molar excess of dithionite per mole of nitrotyrosine in 0.05 M Tris buffer at pH 8.0. After a minute of continuous stirring, the reaction was quenched by eluting the reaction mixture through a G-25 column (2.5 X 20 cm) with 0.1 M acetate buffer. The reduced protein fraction was collected, dialysed and lyophilized.

2.2.1.4. Purification of Aminated Derivatives

The reduced RNase is contaminated with unreduced nitrated RNase. Further separation of the aminated derivatives from the nitrated derivatives was accomplished by chromatofocussing. A column (1 X 20 cm) was prepared with PBE 118 resin (Pharmacia). The column was equilibrated with start buffer (0.025 M triethylamine) at pH 11.0. Diluted ampholytes (pH 8-10.5) were used as the eluent (1:45 dilution of the total volume). The aminated protein was dissolved in 2 mL of the eluent at pH 8.0 and eluted through the column. Pure aminated RNase obtained was dialysed, frozen and lyophilized.

The purity of the aminated derivatives was checked by analytical IEF (Figure 2)

2.2.2. CHARACTERIZATION

2.2.2.1. Analytical IEF

The homogeneity of the nitrated and aminated derivatives was determined by analytical IEF. Preformed acrylamide plates (pH 8-10.5, Serva Corp.) were used. The anode buffer was a mixture of 0.83 g of asparatic acid and 0.92 g of glutamic acid in 250 mL. The cathode solution contained 5 g of glycine in 250 mL of water. Protein samples (3-5 µL) were applied to the gel plates with concentrations in the 2-5 mg/mL range. The plates were focussed at 0°C for 3-4 hrs. at 3-4 watts constant power. Plates were fixed with 20% trichloroacetic acid (w/v) for 20 minutes and stained with Coomassie blue.

2.2.2.2. Catalytic activity

Relative catalytic activity of the aminated derivatives was compared to that of unmodified RNase. The procedure of Crook et al.(1960) (55) for the spectrophotometric assay of unmodified RNase was followed. Cytidine 2',3' cyclic monophosphate was used as the substrate. The kinetics of hydrolysis of cytidine 2',3'cyclic monophosphate were monitored by a change in absorbance at 284 nm, at pH 7.0, 18°C in 1 mL of 0.1 M Tris-Nacl buffer. The enzyme

concentration was 72.5 μM. A standard curve was prepared with unmodified RNase. Initial velocities were determined for different concentrations of the enzyme (0.0725-2.5 μM), the substrate concentration being constant (0.31 μM).

Identical concentrations (72.5 μM) of the two aminated RNases were prepared and their activity was directly compared to the rate of reaction of unmodified RNase.

2.2.2.3. Fluorescence emission spectra

Fluorescence emission spectra for $C^{\varepsilon 115-NH_2}$ RNase and $C^{\varepsilon 115,76-NH_2}$ RNase and unmodified RNase A were examined at pH 6.0 (0.033 M acetate) and pH 3.0 (0.033 M formate). The optimal activation for the aminated derivatives was at 288 nm and the excitation wavelength for unmodified RNase was 280 nm. The emission spectra for both the derivatives was scanned over a wavelength range of 310-500 nm. The concentration of each sample was 35 μM. Both aqueous and 35% methanol (v/v) aqueous/methanol solvents were employed.

2.2.2.4. Quantum yields

The quantum efficiency of the aminated derivatives was determined with respect to quinine sulfate and aminotyrosine.

The solutions of quinine sulfate, amino tyrosine, $C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase were prepared such that all the samples had the same absorbance as 2 X 10^{-4} M $C^{\varepsilon 115\text{-}NH_2}$ RNase at their excitation maxima. Sample solutions were prepared in 1 mL of 0.033 M acetate buffer. Emission spectra for all the solutions were taken at 18°C and -15°C for aminated derivatives and amino tyrosine. The quantum efficiency of amino tyrosine and the aminated derivatives was calculated by comparing the area under the emission peak to that of quinine sulfate. The formula used to calculate the quantum efficiency was :

$$\varphi_{ukn} = \varphi_{kn} \frac{(Abs_{kn})}{(Abs_{ukn})} \frac{(Flu_{ukn})}{(Flu_{kn})} \frac{(Q_{std})}{(Q_{ukn})} \frac{n_2}{n_1}$$

2.2.2.5. Molar absorptivities

Molar absorptivities of the aminated RNases were calculated at pH 6.0, -15°C and 18°C. Protein solutions of 2 X 10^{-4} M were prepared in 1 mL 0.033 M acetate buffer. Absorption spectra were scanned from 190 nm-800 nm. Molar absorptivities were calculated at 278 nm and 288 nm.

2.2.3. REFOLDING KINETICS

The kinetics of the refolding of $C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase were examined at -15°C and 10°C by following the fluorescence emission at 400 nm with excitation at 288 nm. Since the emission maxima for the aminotyrosines is 400 nm and the unmodified tyrosines emit at 305 nm, emission from aminotyrosines can be monitored independently.

In the subzero refolding experiments, an aliquot of 50 µl of protein solution (72 µM) was taken in a gas tight microsyringe (Hamilton) and incubated for 10 minutes at 70°C in a water bath. Previous NMR experiments (Biringer & Fink, 1982a) had revealed that these conditions result in an apparent complete unfolding of RNase A. The unfolded protein was then injected into a cuvette containing 1 mL of cryosolvent (35% methanol, pH 6.0, 0.033 M acetate) held at -15°C ± 0.2°C. The buffer was immediately stirred following the injection of protein and the time dependent changes in fluorescence were recorded. Experiments were terminated when no further change in the fluorescence was observed for at least 1000 seconds.

Refolding kinetics of the aminated derivatives were also monitored at 10°C in 0.05 M cacodylate buffer, pH 5.5. In

these experiments, the protein (72 μM) was unfolded in 5 M urea at pH 2.2. Refolding was initiated by injecting an aliquot of 50 μl of unfolded protein solution 1 mL cuvette containing 0.05 M cacodylate buffer, previously equilibrated at 10°C. After a short mixing time, time dependent fluorescence changes were monitored.

2.2.3.1. Kinetic data analysis

The data from all experiments was analyzed by using Excel™ spreadsheet and NFIT™, a Curve Fitting Program.

The multiphasic kinetics were analyzed in a step-wise fashion. In each case, the slowest phase was analyzed as a single exponential. The best fit data was then subtracted from the entire data set. This process was repeated on the resulting data set. The "stripping" procedure continued until only a single exponential remained. The measured rate constants for all the phases differed by a factor of about 10. Kinetic analysis was also carried out by using a curve-fitting procedure for multiple exponential processes by a mean least square fit procedure. Similar results were obtained with both the methods.

2.2.3.2. Amplitude calculations

The amplitude of each kinetic phase is presented as a percentage of the amplitude difference between that of native protein and protein unfolded in 5 M guanidine

hydrochloride. The amplitudes of native and unfolded protein were obtained under each of the solvent, pH and temperature conditions employed in the folding experiments.

2.2.3.3. Condition dependence of the slowest phase

In order to determine if the amplitudes observed for the slowest phase were dependent on the unfolding conditions, additional refolding experiments were performed on $C^{\varepsilon 115-NH_2}$ RNase. The protein was unfolded in 5 M urea, 5 M guanidine hydrochloride and thermally unfolded at 70°C. Refolding kinetics were examined by fluorescence at 288 nm/400 nm at 10°C in 0.05 M cacodylate buffer.

2.2.4. UNFOLDING ASSAY

To examine the cis-trans isomerization about Pro-114, an unfolding assay was performed on the aminated derivatives. An aliquot of 100 µL of 0.72 mM stock solution was thermally unfolded at 70°C in a water bath for 10 minutes. The refolding reaction was initiated by injecting the unfolded protein into 150 µL of the refolding buffer (35% methanol, 0.033M acetate) at pH 6.0 which was previously equilibrated at -15°C. At regular intervals, 100 µL aliquots of the refolding protein were injected into 900 µL of 5 M guanidine

hydrochloride at pH 6.0. The unfolding of the protein was monitored by fluorescence emission at 400 nm with excitation at 288 nm. The resulting kinetics were analyzed via a least mean square fit to a semilog plot and the standard stripping procedures.

2.2.5.CONTROL EXPERIMENTS

Two different types of control experiments were performed on the aminated derivatives. The overall stability of the fluorometer output and that of native protein was examined by continously monitoring the fluorescence output over a time course of about 5400 seconds to 7200 seconds. Since it is known that aminotyrosines can be oxidized to nitrotyrosines under appropriate conditions. Control experiments were performed to determine if any oxidation occurred during the refolding process and its dependence on the conditions employed in the experiments.

RESULTS AND DISCUSSION

3.1. CONTROL EXPERIMENTS

Control experiments were run to check the baseline stability for the refolding experiments. Solutions with identical concentrations of amino tyrosine, unmodified RNase and aminated derivatives of RNase were prepared. Experiments were conducted at pH 6.0 in acetate buffer and pH 3.0, in 0.033 M formate buffer at -15°C to establish the stability of fluorescence emission. The base-lines appeared to be stable at pH 6.0 for all the samples. However, at pH 3.0, a gradual decrease in fluorescence emission was observed for aminotyrosine and aminated derivatives. This decrease was apparently much greater than that observed for the actual refolding experiment (see below). Moreover, when the pH of the above sample solution was raised to a higher value (8.0), the absorbance spectra indicated that nitrotyrosine was being produced. Since it is known that aminotyrosines are susceptible to oxidation under appropriate conditions, a set of control experiments was performed to examine the condition dependence of this process.

An experiment was performed to determine if oxygen was the source of the process. Oxygen was bubbled into a freshly prepared aminotyrosine solution at pH 3.0 continuously for 3-4 hours. The color of the solution gradually changed yellow

indicating the formation of nitrotyrosine. In a second experiment, nitrogen was purged into the native protein solutions and aminotyrosine solution at pH 3.0 for about an hour and then the stability of the fluorescence emission was tested. The stable fluorescence output indicated that oxygen was the cause of oxidation..

Having obtained the information that oxygen was responsible for the oxidation process, refolding experiments at pH 3.0 were initiated after purging nitrogen into both the protein stock solution samples and the refolding buffer. However, the refolding experiment exhibited the same behavior; a continuous decrease in the fluorescence emission was observed, which was much greater and faster than that expected for an actual refolding experiment. This suggested that the unfolded protein at pH 3.0 was more susceptible to the oxidation process.

The fluorescence emission for the native protein at pH 6.0 was stable, indicating that no oxidation occurs under these conditions. Additional controls were performed to check if the unfolded protein was susceptible to any oxidation during the refolding process at pH 6.0. Aliquots of 50 μL of native protein (0.30 mM) and thermally unfolded protein (at 70°C for 10 minutes) at pH 3.0 were injected into 1 mL of pH 6.0 buffer at room temperature. After allowing sufficient time for the protein to refold under both the conditions, the fluorescence emission was monitored for both of the

solutions. Similar intensities were observed for both the samples. Later, the pH of the solution was changed to 8.0 and the absorbance spectrum measured. No peak corresponding to nitrotyrosine was observed, indicating that incubation at pH 3.0 and elevated temperatures are not responsible for oxidation.

A set of control experiments was performed to determine if the oxidation was photocatalyzed. Samples of aminotyrosine, native $C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase unfolded in 5 M guanidine hydrochloride were incubated at 15°C in 35% methanol at both pH 3.0 and pH 6.0 under constant irradiation at 288 nm in the fluorometer cell compartment. The fluorescence output at 400 nm was recorded to monitor any oxidation event. Only the samples at pH 3.0 showed any evidence of oxidation.

All the protein stock solutions for the refolding experiments were prepared in pH 3.0 buffer to ensure complete unfolding. Experiments were performed to confirm the stability of the protein at pH 3.0 in the native state. Protein stock solutions were prepared in pH 3.0 buffer. Fluorescence intensity was measured as a function of time for each of the sample solutions at room temperature. The protein appeared to be stable with no change in the fluorescence intensity for at least a week.

Since freshly prepared stock solutions were used in the refolding experiments, it can be concluded that no oxidation

was observed in pH 6.0 refolding experiments.

3.2. DISCUSSION

3.2.1. PREPARATION OF $C^{\varepsilon 115\text{-}NO_2}$ RNase AND $C^{\varepsilon 115,76\text{-}NO_2}$ RNase

Specific nitration of tyrosine residues in RNase by a reaction with TNM was first employed by Beaven and Gratzer (55). However, a maximum of three nitrated tyrosines was produced under the reaction conditions. It was in 1977 that Van der Zee had shown that tyrosyl residues in RNase exhibit specific reactivity towards TNM in the order Tyr-115 > -76>-73.

Experiments performed by Biringer et al.(41) to determine the conditions for nitration indicated that a thirty fold molar excess of TNM with a reaction time of 20 minutes produced predominantly singly ($C^{\varepsilon 115\text{-}NO_2}$ RNase) and doubly ($C^{\varepsilon 115,76\text{-}NO_2}$RNase) nitrated derivatives. The conditions used in this study to prepare the nitrated derivatives were similar to those employed by Biringer et al. (41).

The nitration of tyrosine residues by TNM proceeds via a free radical mechanism and results in the production of covalently cross-linked dimers. Experimental observation revealed that a reduction in protein concentration reduces the amount of dimer production. After removal of covalent

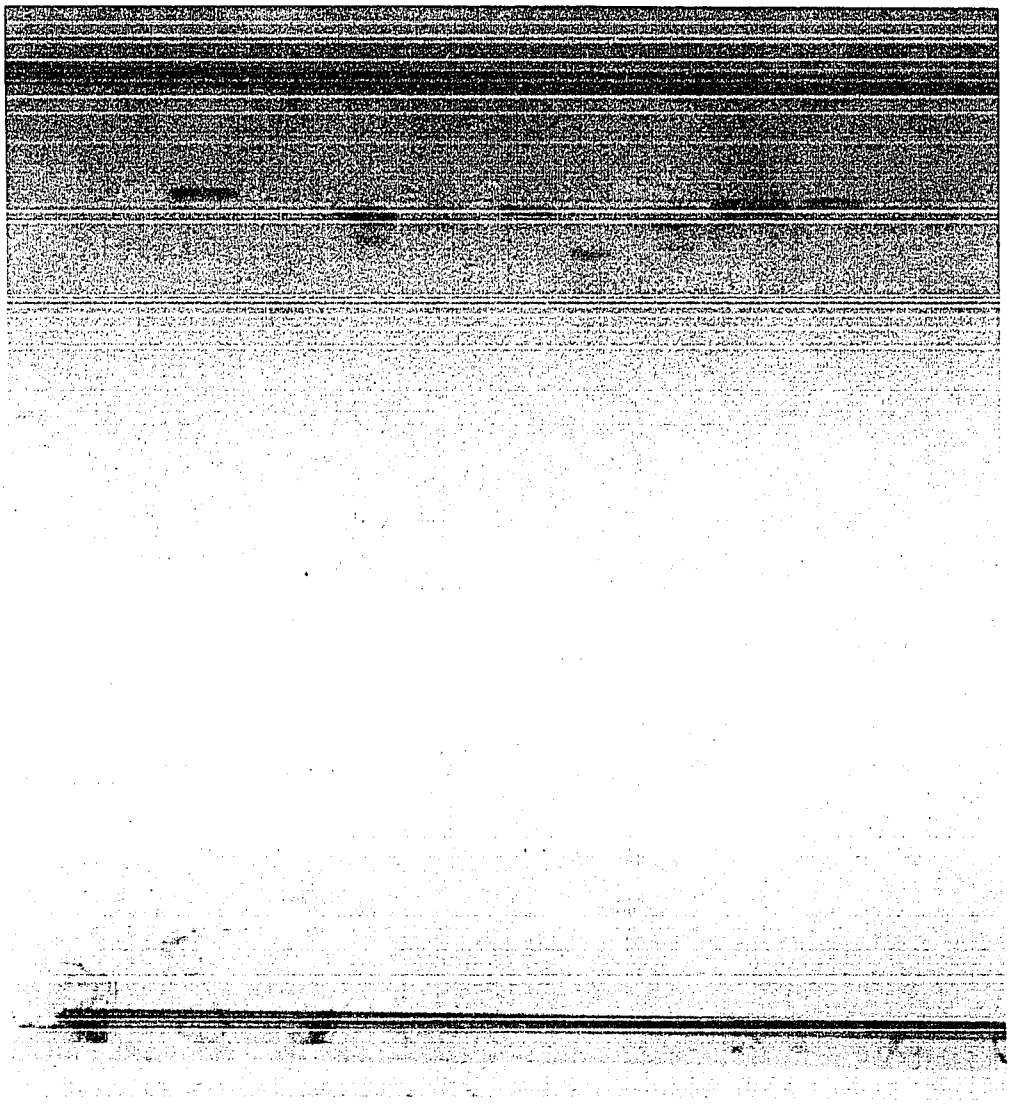

FIGURE 2: Analytical IEF of aminated derivatives of RNase A, pH 3-10. Lanes from left to right : 1. RNase, 2. Markers 3. $C^{\varepsilon 115\text{-}NO_2}$ RNase, 4. crude product from the reduction of $C^{\varepsilon 115\text{-}NO_2}$ RNase, 5. purified $C^{\varepsilon 115\text{-}NH_2}$ RNase, 6. $C^{\varepsilon 115,76\text{-}NO_2}$ RNase, 7. crude product from the reduction of $C^{\varepsilon 115,76\text{-}NO_2}$ RNase, 8. purified $C^{\varepsilon 115,76\text{-}NH_2}$ RNase, 9. RNase.

dimers by gel filtration, the nitrated derivatives of RNase were purified by isoelectric focussing.

Three well separated yellow colored bands were produced. The homogeneity of these derivatives was tested by analytical isoelectric focussing (Figure 2). Single bands were observed for each derivative indicating that they were pure and homogeneous. The yields are as follows:

$C^{\varepsilon 115\text{-}NO_2}$ RNase = 28-32% and $C^{\varepsilon 115,76\text{-}NO_2}$ RNase = 25%.

3.2.2. PREPARATION OF $C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase

The reduction of nitrated tyrosines in RNase A to the corresponding aminated tyrosines by a reaction with dithionite ion was first studied by Seagle and Cowgill (46). The studies confirmed the preferential reactivity of the tyrosine residues towards chemical modification in the order suggested by Van der Zee et al. for nitration reaction. However, the fluorescence properties of the aminated tyrosines were different than those observed for unmodified tyrosines. The excitation and emission maxima for unmodified tyrosines is 280/305 nm. The emission wavelength for the aminated tyrosines is shifted to 380-400 nm with optimal excitation at 288 nm. It was also suggested that the fluorescence emission from aminated tyrosines was more sensitive to the environment than the unmodified tyrosine. As

a consequence, fluorescence efficiency and wavelength of emission of the aminotyrosyl residue should depend on the environment of the modified residue in a protein and the spectral properties should permit differentiation between aminated and unmodified tyrosyl residues.

Individual nitrated derivatives were subsequently reduced to their corresponding aminated derivatives by a reaction with dithionite ion. Preliminary experiments were performed to determine the conditions for the amination reaction. The experiments indicated that 75 fold molar excess of dithionite ion was necessary for every mole of nitrated tyrosine. The products obtained from both the derivatives on amination were tested by analytical isoelectric focussing. The products from both the derivatives showed the presence of some unreduced material (Figure 2). Further purification of the aminated derivatives was accomplished by chromatofocussing. The homogeneity was confirmed by analytical isoelectric focussing (Figure 2).

3.2.3. CHARACTERIZATION

In order to evaluate whether the aminotyrosyl derivatives were effective fluorescence probes of protein structure, the derivatives were further characterized by catalytic activity, fluorescence emission spectra, quantum yields and molar absorptivities.

The enzymatic activity of the two derivatives towards

Cytidine 2′,3′ cyclic monophosphate is given in Table 1. The relative activities have been compared to nitrated derivatives and unmodified RNase A. Tyr-115 and Tyr-76 are solvent exposed and far away from the active site. Hence one would expect that chemical modification would not affect the activity. The results for the nitrated and aminated Tyr-115 indicate that the modification has little effect on the activity of RNase.

However, the activity appears to have decreased for $C^{\varepsilon 115,76\text{-}NH_2}$ RNase derivative. The exhaustive procedure involved in preparation or the presence of bulky nitro and amino groups could have caused slight alterations in the local structure. Since nitrotyrosine-76 and aminotyrosine-76 have similar activity, the use of dithionite ion in the preparation of the amino derivatives probably did not alter the protein structure significantly. Since the ^1H NMR spectra of the nitrated derivatives and RNase are similar, any structural changes accompanying the modifications must be minor (50).

TABLE 1: RELATIVE CATALYTIC ACTIVITIES OF RNase, AMINATED AND NITRATED DERIVATIVES

RNase A	100%
$C^{\varepsilon 115\text{-}NH_2}$ RNase	97%
$C^{\varepsilon 115\text{-}NO_2}$ RNase	98%
$C^{\varepsilon 115,76\text{-}NH_2}$ RNase	88%
$C^{\varepsilon 115,76\text{-}NO_2}$ Nase	92%

Fluorescence emission of aminotyrosines is sensitive to pH and the nature of the solvent. The results in Figure 3 and Figure 4 show the effect of the pH. The difference in the fluorescence intensities is attributed to the change in the ionic state of the amino group. At pH 6.0, the peak at 390-400 nm was ascribed to emission from free base form ($-NH_2$). But the emission at 310 nm at pH 3.0 is due to the cationic($-NH_3^+$) form and was attributed to proton dissociation from the excited singlet state of the cationic form with a shoulder appearing at 360-400 nm (48).

The emission maxima for $C^{\varepsilon 115-NH_2}$ RNase was observed at 400 nm. However, two overlapping bands were observed for $C^{\varepsilon 115,76-NH_2}$ RNase, one appearing at 340-360 nm and a shoulder at 400 nm. The peak at 340-360 nm is probably due to Tyr-76 and the shoulder at 400 nm is due to Tyr-115.

FLUORESCENCE EMISSION SPECTRA:

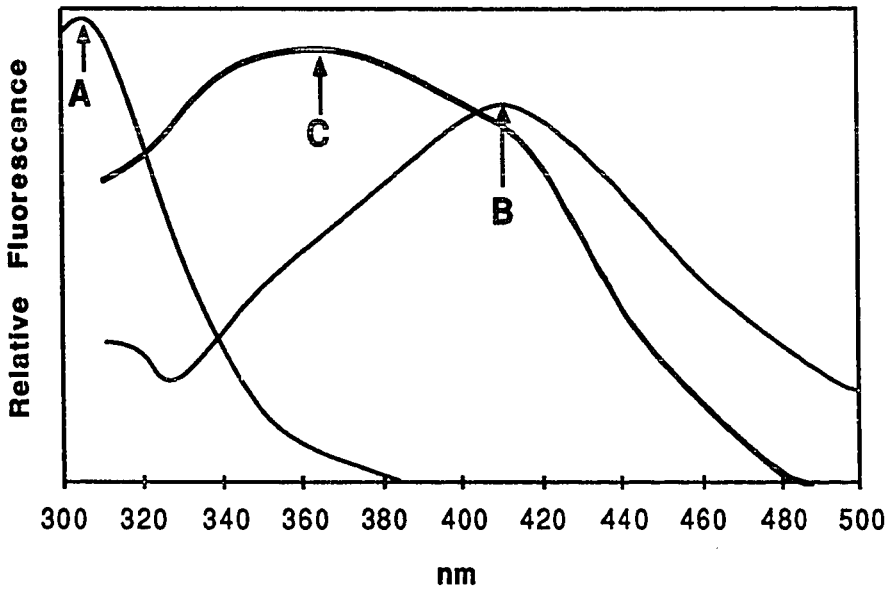

FIGURE 3 : Fluorescence spectra for 35 μM RNase, and aminated derivatives, $C^{\varepsilon 115\text{-}NH_2}$ RNase, and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase at pH 6.0 in 0.033 M acetate buffer. Excitation was at 280 nm for unmodified RNase and 288 nm for the aminated RNases. (A): RNase at 1/10 of the scale used for the aminated derivatives. (B): $C^{\varepsilon 115\text{-}NH_2}$ RNase. (C): $C^{\varepsilon 115,76\text{-}NH_2}$ RNase.

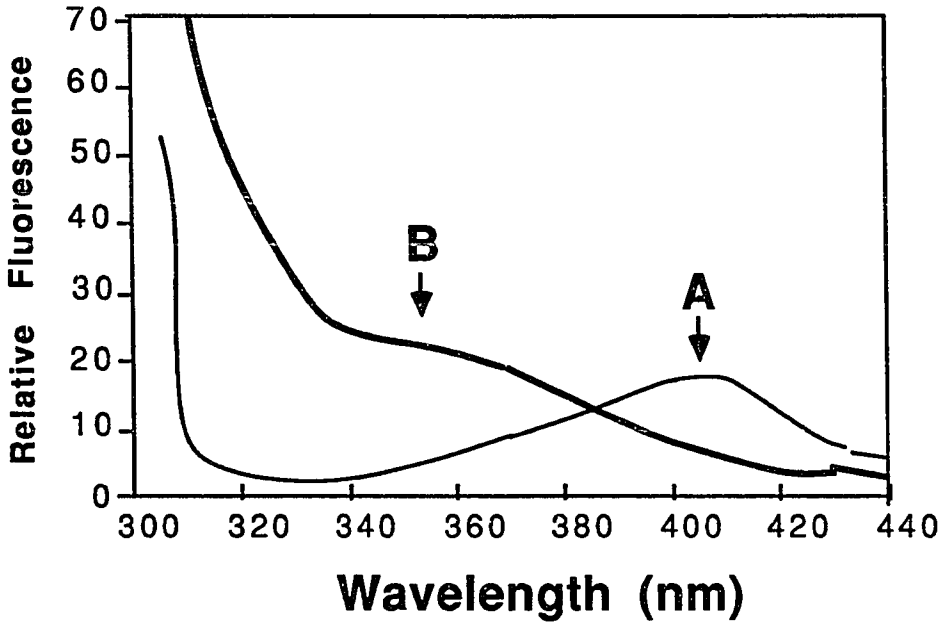

FIGURE 4: Fluorescence emission spectra for $C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase at pH 3.0 in 0.033 M formate buffer. Aminated derivatives were excited at 288 nm.(A).$C^{\varepsilon 115\text{-}NH_2}$ RNase; (B). $C^{\varepsilon 115,76\text{-}NH_2}$ RNase.

The quantum yields for $C^{\varepsilon 115\text{-}NH_2}$ RNase , $C^{\varepsilon 115,76\text{-}NH_2}$ RNase and amino tyrosine are shown in Table 2. Quinine sulfate in 1 M H_2SO_4 was used as reference to calculate the fluorescence efficiency of free aminotyrosine and aminated derivatives. The relative areas under the emission peaks were compared to that of quinine sulfate. Corrections for differences in refractive indices have been made.

The lower fluorescence efficiency of aminotyrosines in proteins compared to that of free aminotyrosine can be attributed to several factors, such as strong quenching by water or a peptide carbonyl group, or quenching of the aminotyrosyl groups by carboxylate groups in the surrounding environment by forming a hydrogen bond between the carboxylate group and the phenolic hydroxyl group.

It was also observed that the fluorescence efficiency for free amino tyrosine and the aminated derivatives increased with decrease in temperature. This is expected at lower temperatures because of the reduced collisions.

TABLE 2: QUANTUM YIELDS FOR THE AMINATED DERIVATIVES:

conditions	aminotyrosine	$C^{\varepsilon 115-NH_2}$	$C^{\varepsilon 115,76-NH_2}$
Aqueous, 18°C		0.0044	0.0042
35% Methanol			
18°C	0.073	0.015	0.014
-15°C	0.22	0.034	0.037

* Aminotyrosine and aminotyrosyl RNase quantum yields are based on the relative integrated areas of the spectral bands as compared to that for quinine sulfate (1 M H_2SO_4) and the accepted quantum yield of 0.55 for quinine sulfate.

The molar extinctions for the aminated derivatives were calculated at 278 nm and 288 nm. The results are shown in Table 3. The molar extinctions are essentially the same at 278 nm under all the conditions and are larger than that of unmodified RNase (9800 AU/M). This suggests that the modification could have altered the molar absorbance and/or aminotyrosine-76 contributes very little to the absorbance at this wavelength. The molar extinction at 288 nm for $C^{\varepsilon 115,76-NH_2}$ RNase is lower than that of $C^{\varepsilon 115-NH_2}$ RNase, indicating that the modification at Tyr-76 alters the molar absorbance of aminotyrosine-115 and probably results in a small structural change.

3.2.4. REFOLDING KINETICS

Since the emission peak from the aminotyrosyl residues is well resolved from that of the remaining aromatic residues, the environment about a specific aminotyrosine residue can be monitored without the interference from other tyrosines. Since both the derivatives contain aminotyrosine-115 and since Pro-114 is followed by a modified tyrosine residue, this particular wavelength would serve to monitor the conformational changes around Tyr-115 which would reflect the isomerization state of Pro-114.

In the present study, the kinetics of refolding were measured at -15°C and 10°C. The lower temperature refolding

TABLE 3: MOLAR EXTINCTIONS OF THE AMINATED DERIVATIVES.

conditions	wavelength	$C^{\varepsilon 115\text{-}NH_2}$	$C^{\varepsilon 115,76\text{-}NH_2}$
Aqueous, 18°C	278nm	12,400	12,500
	288nm	10,200	8,800
35%Methanol			
18°C	278nm	13,500	13,600
	288nm	11,200	10,200
-15°C	278nm	12,400	12,400
	288nm	10,300	9,300

experiments were performed in 35% methanol, pH 6.0. These conditions were chosen so that the results obtained could be compared to those reported by Biringer et al.(41). The higher temperature refolding experiments were performed at pH 5.5 to compare to those results reported by Lin and Brandts (53). In all cases, refolding was monitored by fluorescence emission at 400 nm with excitation at 288 nm.

The rates and amplitudes observed for both the aminated and corresponding nitrated derivatives (41) at -15°C are shown in Table 4. The data indicate that the aminated derivatives provide a more sensitive probe for structural changes than the corresponding nitrated derivatives. In all the cases, kinetics for the aminated derivatives were found to be triphasic and slower than those observed for nitrated derivatives.

The rate constants and amplitudes observed for both the aminated derivatives were similar. This indicates that the aminotyrosine-76 contributes little towards the observed kinetic phases. Since Tyr-76 is more buried than Tyr-115, its modification is more apt to result in structural alterations. The fact that similar kinetics were observed for both the derivatives indicates that the modification of Tyr-76 probably did not alter the folding pathway and strongly suggests that the modification of Tyr-115 probably did not alter the pathway as well.

In light of these results, the slowest process observed in the kinetics is probably associated with the isomerization

TABLE 4: FOLDING KINETICS FOR THERMALLY UNFOLDED RNase A AND DERIVATIVES AT -15°C IN 35% METHANOL, 0.033 M ACETATE BUFFER, pH 6.0:

signal	K_1 (AMP)[a]	K_2 (AMP)	K_3(AMP
RNase[b]	$1.7X10^{-3}$(19)	$3.1X10^{-4}$ (5)	-----
115-NH_2	$1.4X10^{-3}$(15)	$5.3X10^{-4}$(-23)	$6.1X10^{-5}$(57)
115,76-NH_2	$1.7X10^{-3}$(14)	$3.5X10^{-4}$(-29)	$7.2X10^{-5}$(61)
115,76-NO_2[c]	$2.1X10^{-3}$(12)	------	----

[a]The amplitudes shown in the brackets are given as percentage of the total Fluorescence difference observed between the native protein and the protein unfolded in 5 M guanidine hydrochloride.

[b]Data for RNase and nitrated derivatives were taken from reference (30).

[c]Only the slowest of three observed phases is given. Kinetics for $C^{\varepsilon 115-NO_2}$ RNase are faster than any of those listed.

REFOLDING KINETICS FOR $C^{\varepsilon 115\text{-}NH_2}$ RNase, AND $C^{\varepsilon 115,76\text{-}NH_2}$ RNase:

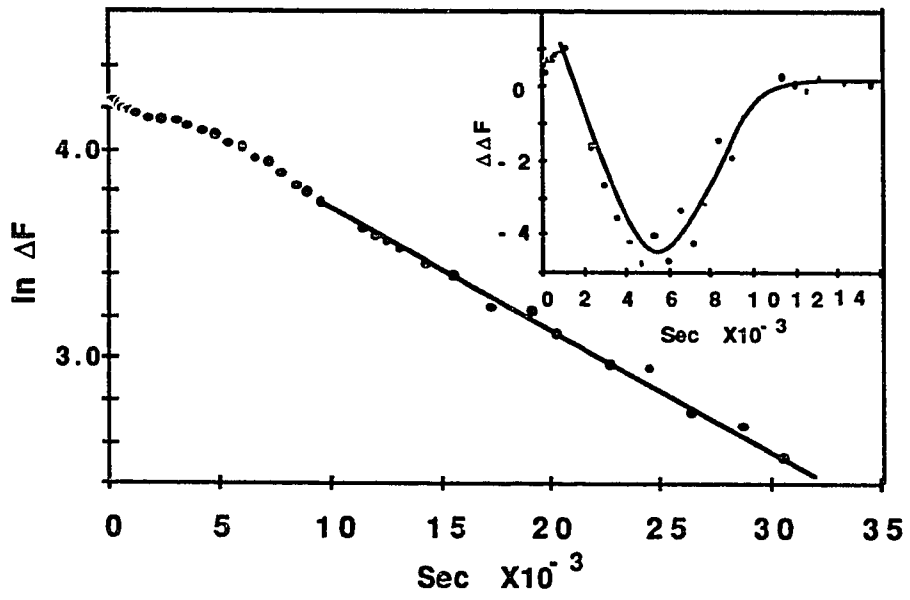

FIGURE 5: Time dependent changes in the refolding of $C^{\varepsilon 115\text{-}NH_2}$ RNase at pH 6.0, in 35% methanol, -15°C. Inset: data after stripping the slowest phase.

of a proline residue.

Since all the kinetics were monitored at 400 nm, which reflects the emission from aminated Tyr-115, the slowest phase could be attributed to the isomerization state of Pro-114. If this is the case, the amplitudes indicate that a greater percent of molecules are involved in the slowest process than reported earlier (53). It does not reveal information regarding the actual cis-to-trans ratio of Pro-114.

The increase in fluorescence associated with the second kinetic phase (Table 4, Figure 5) in the subzero folding experiments for both the derivatives suggests the presence of an intermediate along the folding pathway that is buried and re-exposed during the folding process. The presence of such an intermediate in the absorbance monitored folding of RNase A has been reported earlier (32). A second possible explanation for the existence of this intermediate structure was suggested by Mathews et al.. They observed a similar phase in the folding of tryptophan synthase. However, when the phenylalanine residue adjacent to a tryptophan residue was substituted by alanine, this phase was not observed in the folding experiments. They associate the existence of this phase to exciton coupling between two aromatic residues.

The rate constant for the second phase is consistent with the slowest phase observed for the refolding of unmodified RNase by fluorescence and has been shown to be associated with Pro-93 (32).

As discussed in the introduction, the contribution of Pro-114 towards the folding process is controversial. The specific aim of this project was to examine the contribution of Pro-114. Lin and Brandts have studied Pro-114 by absorbance spectroscopy and isomer specific proteolysis. They observed a small spectroscopic phase which was termed the "ct" phase with an amplitude of 5% of the total possible amplitude. The ISP results also indicated that Pro-114 isomerizes from cis-to-trans at the same rate as the "ct" phase with an amplitude of 5%. Thus, they concluded that Pro-114 was only 5% trans in the equilibrium unfolded species and therefore does not contribute significantly towards the folding process. The following conditions were employed in their experiments: 5 M urea was used as the denaturant, experiments were performed at 10°C in pH 5.5 cacodylate buffer. Refolding was monitored by absorbance at 286 nm where the relative exposure of all the six tyrosines was examined. In the present study, refolding experiments on aminated derivatives were performed using the same conditions except that the kinetics were monitored by fluorescence emission at 400 nm. The results are shown in Table 5 & Figure 6.

Both sets of experiments showed triphasic kinetics. However, the rates for the aminated derivatives were slower than those reported by Brandts et al. and the amplitudes for all the phases observed were greater. The "abortive" intermediate observed at subzero temperature was not observed at all. This suggests that the structural changes responsible

for that particular process are either too fast to be observed at higher temperatures or overlap with decreasing fluorescence events at this temperature.

Brandts ascribes the slow phase in the absorbance monitored kinetics to proline isomerization. We too attribute our slowest phase to proline isomerization.

However, the rate constants and the amplitudes differ, indicating that the two techniques serve to monitor different processes. If we assume the amplitude for the slowest phase is a reasonable representation of the number of unfolded molecules with an incorrect isomerization state of Pro-114, then the ratio of cis/trans isomers present in the unfolded state are different from those reported by Brandts (95:5), suggesting that a greater percent of trans isomers of Pro-114 are present in the equilibrium unfolded species.

TABLE 5 : FOLDING KINETICS FOR UREA UNFOLDED RNase AT 10°C, PH 5.5 IN 0.05 M CACODYLATE BUFFER:

signal	K_1 (AMP)[a]	K_2 (AMP)	K_3(AMP)
RNase[b]	4.3 $X10^{-2}$ (51)	1.4 $X10^{-2}$ (24)	2.5 $X 10^{-3}$ (5)
115-NH_2	3.1 $X10^{-2}$ (16)	4.1 $X10^{-3}$ (35)	1.7 $X10^{-4}$ (50)
115,76-NH_2	3.6 $X10^{-2}$ (12)	5.3 $X10^{-3}$ (40)	2.0 $X10^{-4}$ (45)

[a]The amplitudes shown in the brackets are given as percentage of the total Fluorescence difference observed between the native protein and the protein unfolded in 5 M guanidine hydrochloride.

[b]Data for RNase was taken from reference (54).

REFOLDING KINETICS FOR $C^{\varepsilon 115\text{-}NH_2}$ RNase AND

$C^{\varepsilon 115,76\text{-}NH_2}$ RNase AT 10° C.

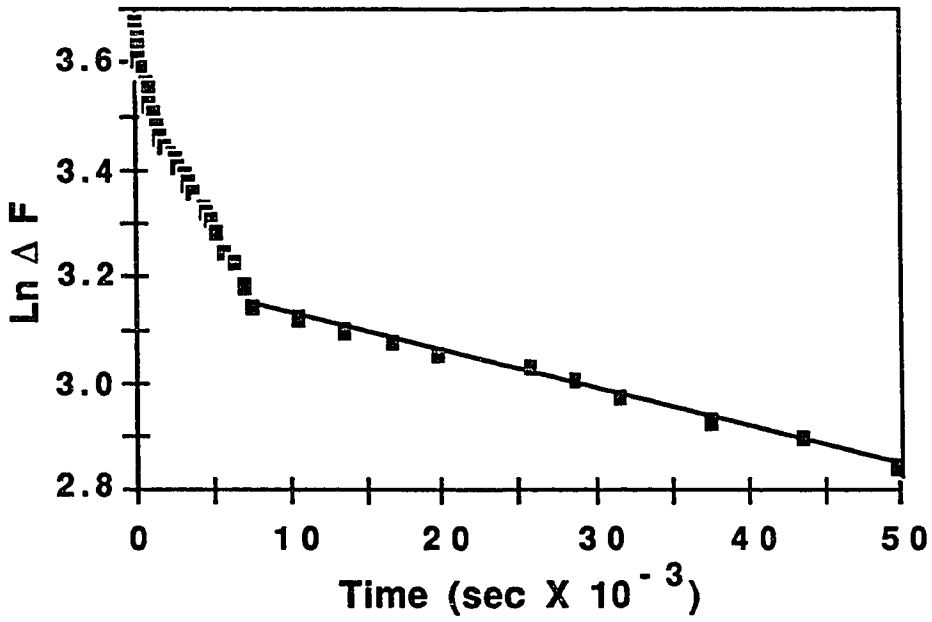

FIGURE 6: Time dependent changes in the refolding of $C^{\varepsilon 115\text{-}NH_2}$ RNase at pH 5.5 in 0.05 M cacodylate ,10°C.

NMR studies on the denatured derivatives of RNase by Biringer (unpublished) indicate 62:38 and 25:75 ratios for the cis/trans isomers for Pro-114 in 5 M guanidine hydrochloride and thermally unfolded protein, respectively. However, the data does not allow the differentiation between which percentage represents the cis or trans isomer. The NMR data by Adler and Scheraga (49) also suggests a 30% cis content for Pro-114 in thermally unfolded protein. These higher percentages are more consistent with our observation than those of Brandts. However, the conditions employed for these experiments were different. Therefore, one cannot rule out the possibility that the isomerization state of proline could be condition dependent.

In order to determine if unfolding conditions define the ratio of cis/trans isomers of Pro-114, additional refolding experiments were performed to determine the condition dependence of the slowest kinetic phase. Three different denaturants were used for this purpose: (1).thermal unfolding at 70°C, (2) 5 M urea, and (3) 6 M guanidine hydrochloride. The refolding was monitored by fluorescence emission at 400 nm at 10°C, in pH 5.5, 0.05 M cacodylate buffer. Table 6 shows the results observed.

The rate constants for all the three phases were similar; however, the amplitudes for the slowest phase were denaturant dependent, which could be a possible explanation for the different ratios observed for the isomerization state of

THE CONDITION DEPENDENCE OF THE SLOWEST PHASE IN THE REFOLDING KINETICS.

TABLE 6: $C^{\varepsilon 115\text{-}NH_2}$ RNase FOLDING AMPLITUDES FOR UREA, THERMAL, AND GUANIDINE UNFOLDED RNase AT 10°C, PH 5.5 IN 0.05 M CACODYLATE BUFFER:

signal	5 M Urea	Thermal	5M guanidine
$A1^a$	8	16	14
A2	35	23	17
A3	50	63	75

[a]The amplitudes are given as percentage of the total Fluorescence difference observed between the native protein and the protein unfolded in 5 M guanidine hydrochloride.

Pro-114. However, the amplitude differences are significantly smaller than those which could account for the differences between this study and the data of Brandts.

3.2.5. UNFOLDING ASSAY

A slow refolding assay developed by Schmid (52) to determine the amount of slow folding species present at different times of unfolding/folding has been discussed in the introduction. It has been shown that the slowest phase in the fluorescence monitored refolding of RNase is associated with the isomerization of Pro-93 and that it is associated with high activation energy (17-20 Kcals/mole). The second kinetic phase observed in the folding of the aminated derivatives has the same rate. Since we expect that all proline isomerizations will be the slowest events, due to the high activation energy, it would be reasonable to assume that the slowest phase observed in our experiments is also associated with the isomerization of a proline and one other than Pro-93.

An unfolding assay was performed by modifying the above procedure to determine the actual cis-to-trans ratio of Pro-114 and also to determine which of the kinetic phases is associated with proline isomerization.

Biphasic kinetics were observed for the unfolding assays. The standard stripping procedures were used to analyze the phases. The rate constants for both the phases differed by a

factor of 5.

The amplitudes for the slowest phase were plotted as a function of sampling time. The plot is shown in Figure 7. The amplitude for the slow phase shows a gradual increase as a function of sampling time. The dependence of these slow unfolding amplitudes on the sampling time indicates the isomerization state of the native proline residues. The data is clearly sigmoidal and thus indicates that this process is strongly coupled to an earlier event.

The amplitude for the faster phase (not shown) increases as a function of sampling time in the first 5000 seconds but gradually decreases at later sampling times. An amplitude profile such as this indicates that it is strongly coupled to a subsequent event. Therefore, this is apparently coupled to the slower process.

The data for the slowest phase was fit to a sequential process using the equation defined by Frost and Pearson (56). For a simple sequential model:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

$$[C] = A_o - A_o \left[k_1(k_2-k_1)\right] \left(e^{-k_1 t} - e^{-k_2 t}\right) + A_o e^{-k_1 t}$$

DOUBLE-JUMP UNFOLDING ASSAYS

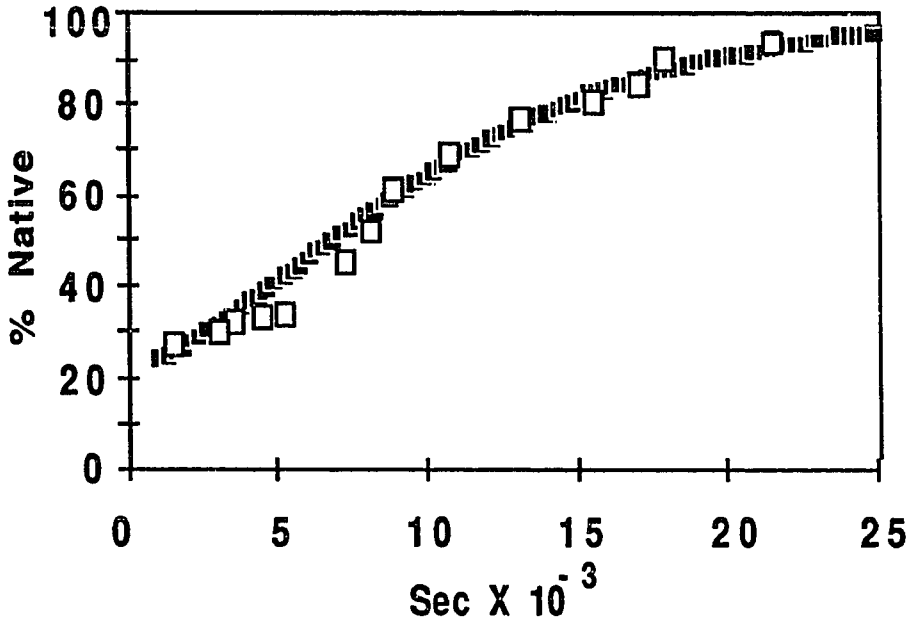

FIGURE 7`: Results of the double-jump assay. The data represents the amplitudes of the slowest phase obtained from the assay as a function of sampling time. The data is sigmoidal and thus indicates that this process is strongly coupled to an earlier one.

where A_o is the initial concentration of "A" at the start of the reaction.

The best-fit intercept indicates that 22% of the molecules are in the trans configuration in the unfolded mixture. This value is closer to the NMR data presented by Adler and Scheraga and that of Biringer, than the 5% reported by Brandts. The rate constants obtained from the fit are similar and have the values between the rates for the 2nd and 3rd phases observed in the folding kinetics. It is difficult to ascertain which kinetic phase in the fluorescence monitored folding is associated with the isomerization process.

There are several possibilities that can explain the apparent similarity in the rates for both the processes and the fact that neither correspond to the rates observed in the folding of the aminated derivatives.

Firstly, the data was fit to a simple sequential model where it was assumed that the amplitude of the slowest phase in the assay represented the formation of the native protein along a simple pathway. If the folding reaction is more complex or the amplitude of the assay's slowest phase is not entirely due to the formation of the native material along one path, the apparent rate constants would represent an average of two or more rates. The sigmoidal data for the slow phase strongly suggests that it is coupled to an earlier process. It is also consistent with two parallel sequential processes; that is, instead of one sequential process there

are two parallel processes which arrive at the same native product independently. Two possible models are as follows:

$A \xrightarrow{k_1} B \xrightarrow{k_2} N$ or $A \xrightarrow{k_1} B \xrightarrow{k_2} N$

$C \xrightarrow{k'_2} N$ $D \xrightarrow{k'_1} E \xrightarrow{k'_2} N$

In each case, the amount of final product is defined by two paths. If k_2 and k_2' are similar, the apparent rate constant would represent an average of the two and hence underestimate the value for the slower process.

Secondly, the presence of a decreasing process underlying an increasing process could also effect the best-fit amplitudes and rates. Since we have observed such a process in the folding reactions, we cannot rule out its presence in the unfolding assay. Overlap of such processes could result in a decrease in the observed amplitudes which would yield an underestimation of the rate constants.

Lastly, in the unfolding assay, the refolding reaction was initiated at -15°C and the sample was injected for unfolding at 10°C. If the effect of the temperature is considered, some folding might occur at 10°C before thorough mixing and subsequent unfolding. As a consequence, more native molecules would be formed earlier than they would have if the temperature were held constant at -15°C. This could result in an underestimation of the actual folding rates. These possibilities are currently under investigaion.

CONCLUSION

The time dependent changes in the properties of the intrinsic spectroscopic probes within a protein molecule provide only a global picture of the folding process. However, chemical modification of existing amino acid side chains allows an independent examination of the modified and unmodified residues and provides information regarding the conformational changes in particular regions of the molecule itself.

Two aminated derivatives of RNase ($C^{\varepsilon 115\text{-}NH_2}$ RNase and $C^{\varepsilon 115,76\text{-}NH_2}$ RNase) have been prepared, purified and characterized by analytical IEF, catalytic activity, emission spectra, quantum yields and molar extinctions.

Singly aminated derivative is essentially as active as the unmodified RNase. A decrease in relative catalytic activity was observed for $C^{\varepsilon 115,76\text{-}NH_2}$ RNase derivative. However, since the activity for both the nitrotyrosine-115,76 and aminotyrosine-115,76 is similar, the reducing agent (dithionite ion) must not have altered the structure. A shift in emission maxima to 360-400 nm was observed for the aminotyrosines.

The quantum efficiency and molar extinctions were found to be temperature dependent. The molar extinction for the

modified derivatives are greater than those observed for unmodified RNase, which suggests that modification alters the absorbance properties. Molar extinctions were lower for $C^{\varepsilon 115,76-NH_2}$ RNase. Since Tyr-76 is more buried than Tyr-115, modification of this residue could have altered the absorbance properties. However, further research is necessary to determine the structural change,if any.

Refolding kinetics were monitored at -15°C and +10°C by fluorescence emission at 400 nm. Multiphasic kinetics were observed under both the conditions. The rate constant for the slowest phase is consistent with the isomerization of proline. Since the kinetics were monitored at 400 nm which reflects the emission from the aminotyrosine 115, it can be assumed that the slowest phase is associated with structural changes in Pro-114. The amplitude observed for the slowest phase is greater than that observed earlier (53), indicating that a greater percentage of molecules are involved in the slowest process.

The increase in fluorescence associated with the second phase in the subzero folding experiments suggests the presence of an intermediate structure along the folding pathway. Whether this is actually associated with structural changes resulting in the presence of an intermediate structure or is the result of an exciton coupling has to be investigated.

The slowest phase has been observed to be denaturant dependent. A condition dependent study on $C^{\varepsilon 115\text{-}NH_2}$ RNase revealed that the amplitudes for the slowest phase are dependent on the unfolding conditions.

An unfolding assay was performed on the $C^{\varepsilon 115\text{-}NH_2}$ RNase derivative. A sigmoidal kinetic phase was observed for the slowest phase suggesting a coupling event between two processes. The data was fit to a sequential process which resulted in similar rate constants for both the processes. The intercept clearly indicates that ≈22% of the proline molecules are trans in the equilibrium unfolded species. But in light of the results obtained, it is difficult to ascertain which of the slow phases observed in the kinetics can be associated with the isomerization of proline residue. Whether the coupling results in a sequential process or if two parallel processes are involved has to be determined. The results are being investigated.

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