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^{1}H,^{13}C and ^{15}N backbone assignments of cyclophilin when bound to cyclosporin A (CsA) and preliminary structural characterization of the CsA binding site

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The backbone ^{1}H, ^{13}C and ^{15}N chemical shifts of cyclophilin (CyP) when bound to cyclosporin A (CsA) have been assigned from heteronuclear two- and three-dimensional NMR experiments involving selectively ^{15}N- and uniformly ^{15}N- and ^{15}N,^{13}C-labeled cyclophilin. From an analysis of the ^{1}H and ^{15}N chemical shifts of CyP that change upon binding to CsA and from CyP/CsA NOEs, we have determined the regions of cyclophilin involved in binding to CsA.

Cyclophilin; Cyclosporin A; NMR

1. INTRODUCTION

Human cyclophilin (CyP) from T lymphocytes is a protein of 165 amino acids and binds specifically to the immunosuppressive drug, cyclosporin A (CsA) [1,2]. Several pieces of evidence suggest that the CyP/CsA complex is the biological effector involved in immunosuppression, possibly by binding to and modulating the effects of a protein involved in T cell activation [3]. Indeed, it has recently been shown that the CyP/CsA complex as well as another immunophilin/immunosuppressant complex (FKBP/FK506) bind to and inhibit the calcium- and calmodulin-dependent phosphatase, calcineurin, which may play an important role in T cell and IgE receptor signaling pathways [4].

In order to understand the structural basis for the interaction between the immunophilins and immunosuppressants as well as the common structural features that may be required for the binding of the immunophilin/immunosuppressant complexes to other proteins, it is important to determine the three-dimensional structures of the immunophilin/ligand complexes. A crystal structure of the FKBP/FK506 complex has been solved [5] and solution structures of FKBP have appeared [6,7]. The conformation of CsA when bound to cyclophilin has been determined [8,9], and very recently, an X-ray crystal structure of a tetrapeptide substrate bound to CyP [10] as well as NMR studies of uncomplexed CyP [11] and CyP bound to a water-soluble CsA derivative [10] have been described. However, the complete three-dimensional structure of the CyP/CsA complex has not yet been reported.

As a first step in determining the complete three-dimensional structure of the CyP/CsA complex by NMR, we have assigned the backbone ^{1}H, ^{13}C and ^{15}N chemical shifts of cyclophilin when bound to CsA from heteronuclear two- and three-dimensional NMR experiments involving selectively ^{15}N- and uniformly ^{15}N- and ^{15}N,^{13}C-labeled cyclophilin. From an analysis of the ^{1}H and ^{15}N chemical shifts of CyP that change upon binding to CsA and from the identification of CyP/CsA NOEs, we have identified those portions of CyP that bind CsA.

Abbreviations: CyP, cyclophilin; CsA, cyclosporin A; FKBP, FK506 binding protein; NOE, nuclear Overhauser effect; HSQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cloning of human cyclophilin and expression in *E. coli* was performed as previously described [12]. Competent *E. coli* strains AG-1 were obtained from Stratagene and DL-39 [13] was obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University. All isotopically labeled amino acids were purchased from Cambridge Isotopes. The isotopically labeled cyclophilin samples were purified as previously described [12] from a prototrophic (AG-1) or polyauxotrophic (DL-39) *E. coli* strain [13] harboring the pBS12 plasmid that overproduced human cyclophilin (~10 mg/l). For [U-^{15}N]CyP and [U-^{15}N,^{13}C]CyP, AG-1 cells were grown on minimal media containing [^{15}N]NH_4Cl and unlabeled or uniformly ^{13}C-labeled glucose (Martek

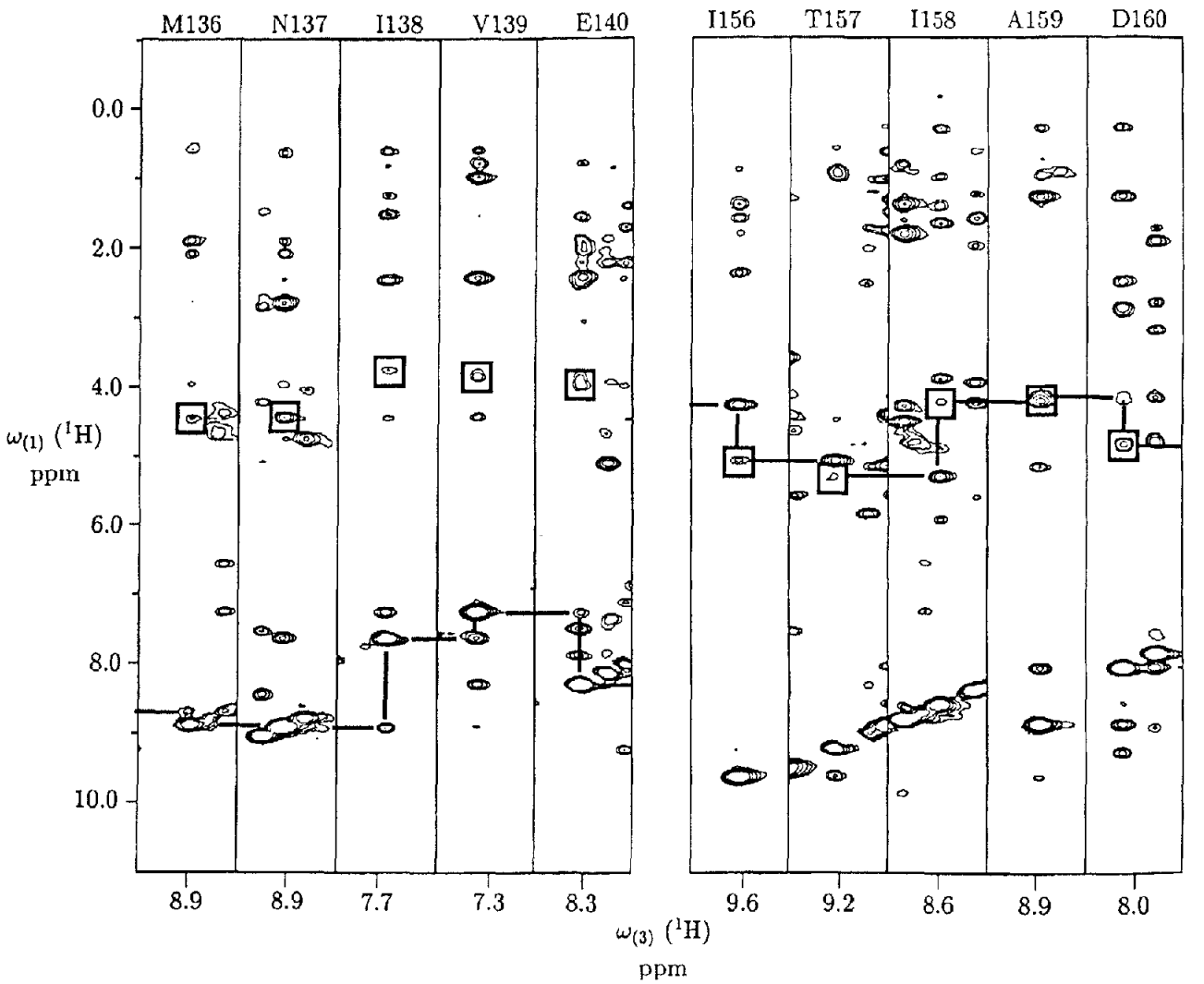

Fig. 1. H(ω_1), ^{1}H(ω_3) cross-sections corresponding to the ^{15}N frequencies (ω_2) of M136-E140 and I156-D160 of a 3D NOESY-HSQC spectrum of [U-^{15}N]CyP/CsA. NH/H$^{\alpha}$ cross-peaks that were observed in a 3D TOCSY-HSQC spectrum of [U-^{15}N]CyP/CsA are indicated by boxes.

Corp.), respectively. [^{15}N-Lys]CyP and [^{15}N-Gly]CyP were obtained from AG-1 cells grown on a defined medium containing a mixture of unlabeled amino acids [14] and uniformly ^{15}N-labeled Lys or ^{15}N-labeled Gly, respectively. [^{15}N-Phe, ^{13}C=O-Leu]CyP, [^{15}N-Ile, ^{13}C=O-Phe]CyP, [^{15}N-Val, ^{13}C=O-Ile]CyP, and [^{15}N-Ala]CyP were isolated from DL-39 containing pBS12 grown on a defined medium [14] containing the appropriately labeled amino acid.

2.2. *NMR sample preparation*

The cyclophilin samples in phosphate buffer (50 mM, pH 6.5), containing NaCl (100 mM) and dithiothreitol (5 mM) in H_2O were concentrated to ~1.3 mM with a centricon YM-10 (Amicon). The CsA/cyclophilin complexes were prepared [15] by gently shaking a suspension of ~1.5 mol equivalents of CsA in a solution of cyclophilin at 6°C for 12 h under argon. Uncomplexed CsA was removed by centrifugation prior to NMR analysis.

2.3. *NMR experiments*

All NMR spectra were acquired on a Bruker AMX600 (600 MHz) NMR spectrometer at 20°C except for the 2D ^{1}H-^{1}H NOESY spectrum which was acquired at 25°C on a Bruker AMX500 NMR spectrometer. The spectra were processed using in-house written software on Silicon Graphics computers.

The 2D ^{1}H-^{1}H NOESY spectrum was acquired on an unlabeled CyP/CsA complex dissolved in ^2H_2O using a sweep width of 7692.3 Hz in ω_1 and 10 000 Hz in ω_2. The data was collected as 320(t_1) × 2048(t_2) complex points using 160 scans per increment and a mixing time of 100 ms.

The ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) spectra [16,17] were acquired using 2048 complex points in t_2 and 512 complex points in t_1, with 96 scans per t_1 experiment. The sweep width was 10 000 Hz in ω_2 (^{1}H) and 2890 Hz in ω_1 (^{15}N).

For the ^{15}N-resolved 3D NOESY-HSQC experiment [17 19] 256(t_1) × 25(t_2) × 1024(t_3) complex points were acquired using spectral widths of 9434 Hz (ω_1, ^{1}H), 2890 Hz (ω_2, ^{15}N), and 10 000 Hz (ω_3, ^{1}H). A 10 ms homospoil pulse was applied during the 50 ms NOESY mixing time and water suppression was accomplished with a 3 ms spinlock pulse using the method of Wüthrich and coworkers [17]. 16 acquisitions were collected per t_1,t_2 experiment for a total experimental time of approximately 6 days.

The ^{15}N-resolved 3D TOCSY-HSQC [20] spectrum was acquired with identical parameters except for 8 scans per t_1,t_2 experiment, 26 complex points in t_2 (^{15}N), and a 5 ms spinlock was used for water suppression. The TOCSY mixing time consisted of a 20 ms MLEV-17 spinlock period (10 kHz) modified for the suppression of rotating frame NOE contributions [21].

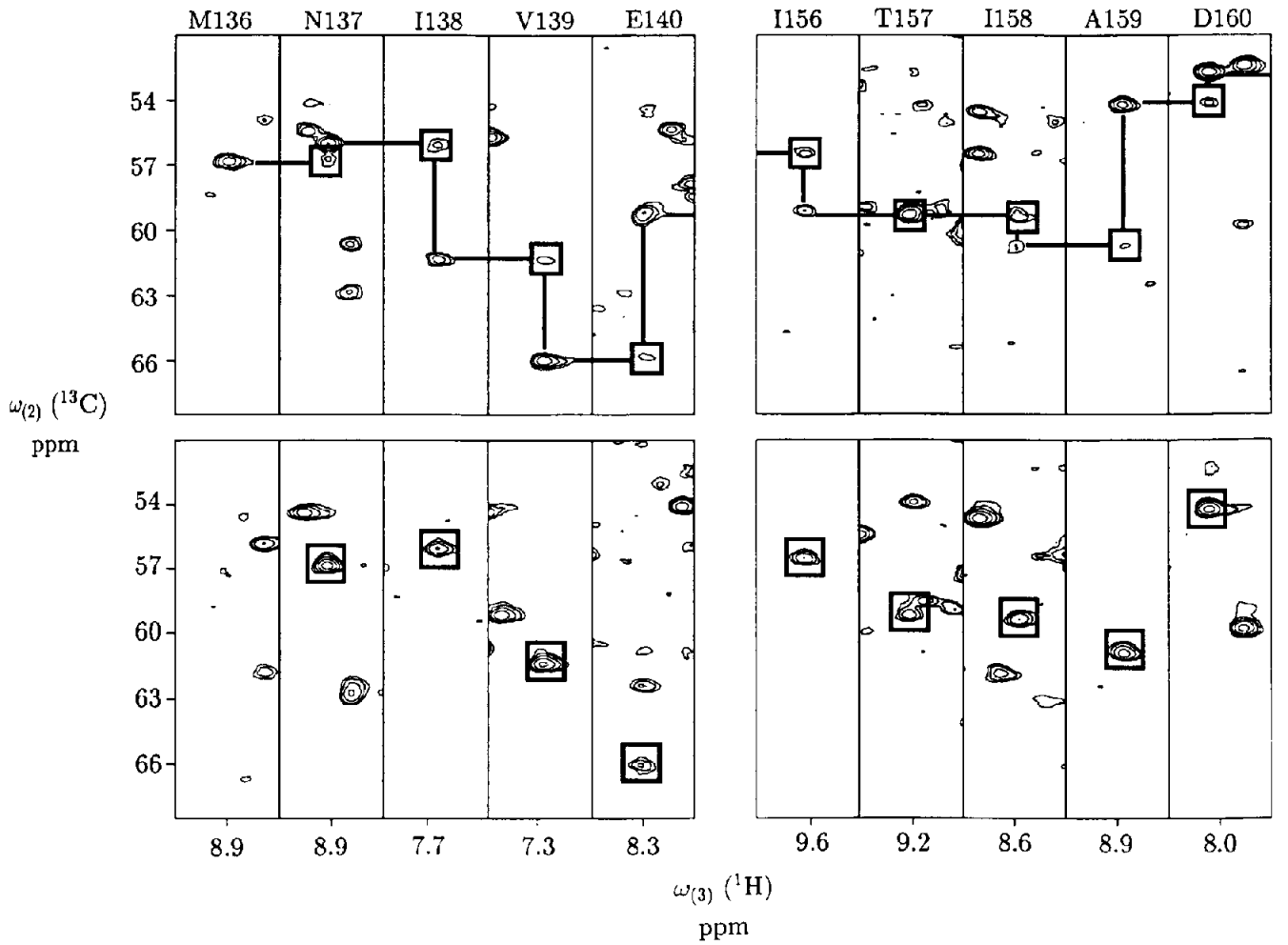

Fig. 2. $C^{\alpha}(\omega_2)$, $NH(\omega_3)$ cross-sections corresponding to the ^{15}N frequencies (ω_1) of M136-E140 and T156-D160 of a 3D HNCA (top) and 3D HN(CO)CA (bottom) spectrum of [U-^{15}N,^{13}C]CyP/CsA. N(i)/C^{α}(i–1)/NH(i) cross-peaks are indicated by boxes. For M136, the N(i)/C^{α}(i–1)/NH(i) cross-peak to G135 is not present in this portion of the spectrum but was observed at 46.1 ppm.

The 3D HNCA experiment [22] was acquired with 32 scans per t_1-t_2 experiment with 42*80*1024 complex points and sweep widths of 2890, 4717, and 10 000 Hz in ω_1(^{15}N), ω_2(^{13}C), and ω_3(^{1}H), respectively. The delay for the evolution of the ^{15}N-$^{13}C^{\alpha}$ coupling was set to 22 ms to minimize relaxation losses.

The 3D HN(CO)CA experiment [23] was acquired with 48 scans/increment under identical sweep widths using 26*64*1024 complex points in ω_1(^{15}N), ω_2(^{13}C) and ω_3(^{1}H), respectively.

The 3D HNCO spectrum [22] was collected as 40*80*1024 complex points using sweep widths of 2890, 2273 and 10 000 Hz in ω_1(^{15}N), ω_2(^{13}C') and ω_3(^{1}H), respectively. 16 scans were acquired per increment for a total acquisition time of 3 days. For all of the triple resonance NMR experiments, water suppression was accomplished using a 3 ms spinlock pulse using the method of Wüthrich and coworkers [17].

3. RESULTS AND DISCUSSION

The ^{1}H, ^{13}C and ^{15}N amide chemical shifts of CyP when bound to CsA were assigned from an analysis of five three-dimensional NMR spectra of [U-^{15}N]CyP/CsA (3D NOESY-HSQC, 3D TOCSY-HSQC) and [U-^{15}N,^{13}C]CyP/CsA (3D HNCA, 3D HN(CO)CA, 3D HNCO) and six two-dimensional HSQC spectra of selectively labeled CyP/CsA samples. Fig. 1 depicts ^{1}H,^{1}H cross-sections (ω_1,ω_3) of an ^{15}N-resolved 3D NOESY-HSQC spectrum of [U-^{15}N]CyP/CsA extracted at different ^{15}N amide frequencies (ω_2). NOEs between CyP amide (ω_3) and other protons appear along ω_2. The chemical shifts of the α-protons that are scalar coupled to the CyP amide protons were obtained in the complementary 3D TOCSY-HSQC spectrum of [U-^{15}N]CyP/CsA extracted at the same ^{15}N amide chemical shifts. The α-protons identified in the 3D TOCSY-HSQC experiment are indicated by boxes in Fig. 1. From an analysis of these two 3D NMR spectra using a strategy that involves the matching of NOE cross-peaks [20,24], the amides of several adjacent amino acids of CyP were identified. For α-helical regions of the protein, adjacent amino acids were identified from NH/NH and other characteristic NOEs [25] as illustrated in Fig. 1 for M136-E140 of CyP. Ambiguities in the analysis of these

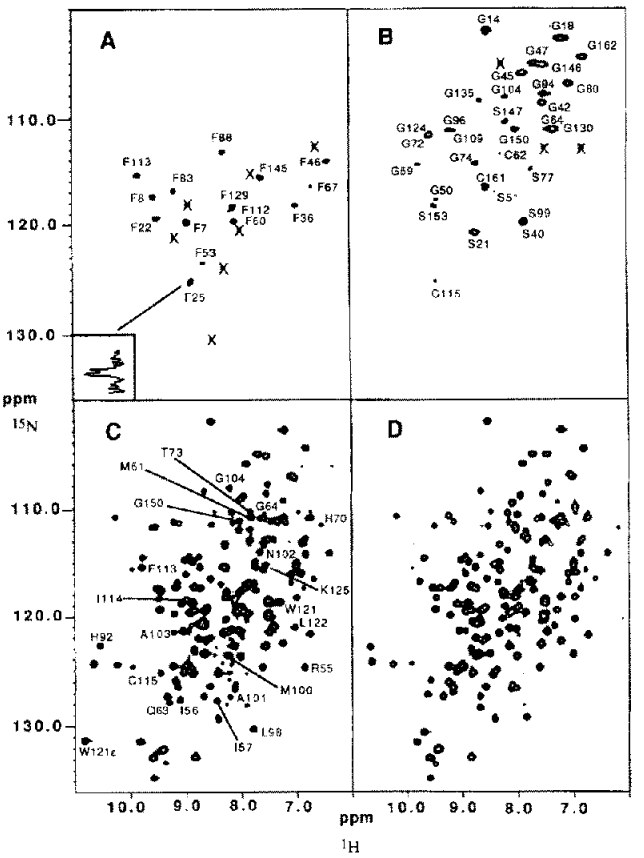

Fig. 3. ^1H, ^{15}N HSQC spectra (^1H, horizontal axis; ^{15}N, vertical axis) of (A) [^{15}N-Phe,^{13}C=O-Leu]CyP/CsA, (B) [^{15}N-Gly,Ser,Cys]CyP/CsA, (C) [U-^{15}N]CyP/CsA, and (D) [U-^{15}N]CyP. The inset in (A) corresponds to a cross-section (vertical axis) of the amide ^1H and ^{15}N frequency of F25. The cross-peaks marked by an 'x' in (A) and (B) were due to sample impurities as they were not observed in (C). CyP amide resonances in (C) that do not match any of the cross-peaks in (D) within the range of ± 0.7 ppm for ^{15}N and/or ± 0.17 ppm for ^1H are labeled in (C).

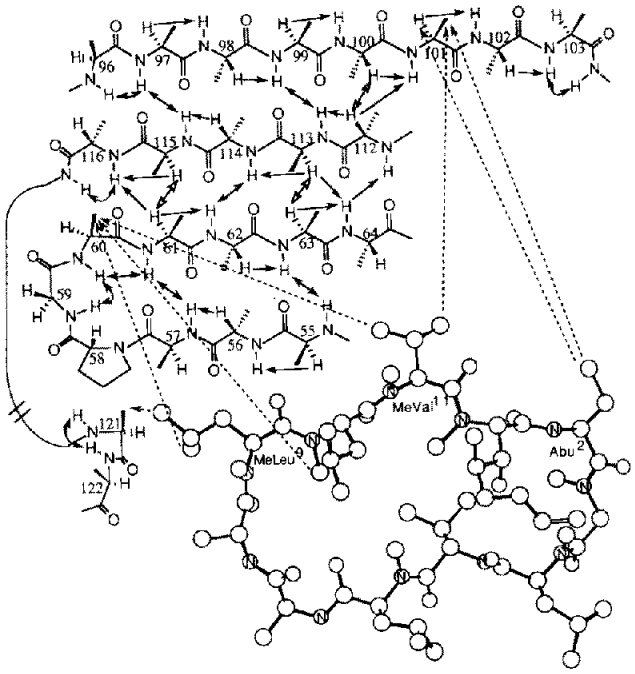

Fig. 4. Secondary structure of the proposed CsA binding site as determined from NOEs observed in a 3D HSQC-NOESY spectrum of [U-^{15}N]CyP/CsA (solid arrows) and ^1H,^1H NOESY spectrum (open-headed arrows) of CyP/CsA. NOEs observed in a ^{13}C 3D HMQC-NOESY spectrum of [U-^{13}C]CsA bound to CyP [9] are indicated by the dashed arrows.

spectra arose for amide protons of adjacent amino acids that resonate at identical chemical shifts (e.g. M136 and N137). In principle, these ambiguities could be resolved from a 3D HMQC-NOE-HMQC experiment [26], or, as described below, from triple resonance 3D NMR experiments [22,23].

The amides of adjacent amino acids for the β-sheet regions of CyP were identified from matching NOEs (primarily NH(i+1)/H$^\alpha$(i)) [25] as illustrated for I156-D160 of CyP (Fig. 1). In some cases, however, the analysis was complicated by the overlap of α-protons, missing cross-peaks in the TOCSY experiment, or the observation of NOEs involving non-adjacent amino acids.

The ambiguities encountered in the analysis of the two ^{15}N-resolved 3D NMR experiments were alleviated using two triple resonance 3D NMR experiments applied to [U-^{15}N,^{13}C]CyP/CsA. In a 3D HNCA experiment (Fig. 2, top), the amide ^1H (ω_3) and ^{15}N (ω_1) chemical shifts of ith residue were correlated to the α-carbons (ω_2) of the i and i–1 amino acids [22]. The N(i)/C$^\alpha$(i–1)/NH(i) cross-peaks that could not be observed in the 3D HNCA spectrum (e.g. Fig. 2, A159, top), that overlapped with the N(i)/C$^\alpha$(i)/NH(i) cross-peaks (e.g. Fig. 2, T157, top), or that could not be unambiguously distinguished from the N(i)/C$^\alpha$(i)/NH(i) cross-peaks by their relative intensities (e.g. Fig. 1, I158, top) were identified in a 3D HN(CO)CA experiment. In this spectrum the ^1H (ω_3) and ^{15}N (ω_1) chemical shifts of the amides (i) are correlated *exclusively* to the α-carbons (ω_2) of the i–1 residues by magnetization transfer through the intervening carbonyl carbons [23], resulting in high sensitivity and an unambiguous distinction between the C$^\alpha$(i) and C$^\alpha$(i–1) cross-peaks.

As illustrated in Fig. 2, data obtained from these two triple resonance 3D NMR experiments complement the ^{15}N-resolved 3D NMR data. For example, although the NH or α-protons overlap in the 3D NOESY-HSQC spectra, these ambiguities may be resolved in the triple resonance 3D NMR experiments (Fig. 2), since they rely on the identification of adjacent residues through the correlation of different chemical shifts (C$^\alpha$). Other complications arising from the observation of NOEs between non-adjacent amino acids could also be resolved in the triple resonance 3D NMR experiments, since unlike the NOE experiment, the triple resonance experiments depend on throughbond couplings between the atoms of the peptide backbone.

From an analysis of the four 3D NMR experiments, many strings of adjacent amino acids of CyP were iden-

tified. In order to obtain the sequence-specific assignments, however, it was important to identify the amino acid spin systems by amino acid type. Since this was difficult to accomplish in the conventional manner from $^{1}H,^{1}H$ scalar correlation experiments, we resorted to selective labeling of the individual amino acids [27,28]. For labeling the Phe, Ile, Val, and Ala residues, a polyauxotrophic strain, DL-39 [13], containing pBS12 was employed to prevent the scrambling of the label. As illustrated for [^{15}N-Phe,^{13}C=O-Leu]CyP/CsA, the amides of the Phe residues were easily identified in the HSQC spectrum (Fig. 3A), and from the $^{15}N/^{13}C$ coupling observed for the unique L24-F25 dipeptide sequence (inset), the amide of F25 was assigned. For ^{15}N-labeling of Gly and Lys, a prototrophic strain (AG-1) was employed as the host strain. Although scrambling of the label occurred when ^{15}N-labeled Gly was part of the culture medium, the additional labeling of the Ser and Cys residues of CyP was useful for identifying the amides of these amino acids (Fig. 3B).

Table I. ^{1}H, ^{13}C, and ^{15}N backbone chemical shifts of CyP when bound to CsA (~1.3 mM) measured at 20°C in phosphate buffer (50 mM, pH=6.5), containing NaCl (100mM) and dithiothreitol (5mM).

Res.	HN	^{15}N	C′	C$^{\alpha}$	H$^{\alpha}$	Res.	HN	^{15}N	C′	C$^{\alpha}$	H$^{\alpha}$
M1	—					K31‡	10.71	123.3	181.0	60.1	4.00
V2						T32	10.28	123.7	177.0	66.9	3.98
N3						A33‡	9.23	124.8	177.4	55.6	4.03
P4	—		174.5	56.9		E34	8.03	117.4	176.7	57.6	4.49
T5	8.79	114.4	174.8	60.4	5.71	N35	7.13	115.2	174.0	56.4	4.03
V6	8.72	120.0	173.1	58.5	5.31	F36	7.02	116.6	178.1	61.1	4.11
F7	8.96	118.9	172.0	54.9	5.88	R37	8.96	120.4	177.3	60.1	
F8	9.54	117.0	174.4	53.0	5.32	A38	8.67	118.5	181.7	53.9	4.04
D9	9.27	123.7	176.6	54.4	5.52	L39	8.22	120.4	178.8	56.9	3.75
I10	9.06	123.7	175.4	57.7	5.20	S40	7.91	118.9	173.8	62.2	4.44
A11	9.65	131.9	174.2	50.5	5.15	T41	7.99	107.7	177.5	62.6	4.27
V12	8.93	117.8	175.9	60.1	4.50	G42	7.58	107.7	177.0	45.4	3.87,3.46
D13	9.87	130.4	175.2	54.5	4.32	E43	8.00	117.8	175.8	58.2	
G14	8.58	101.1	173.1	45.0	4.19,3.41	K44	9.13	117.8	175.9	53.9	4.28
E15	8.06	122.9		52.5	4.78	G45	7.94	104.8	171.9	44.3	4.28,3.55
P16	—		175.4	64.4		F46	6.42	113.3	172.1	53.7	4.60
L17	9.19	125.6	175.3	55.2	4.70	G47	7.74	104.0	170.9	45.2	4.34,2.50
G18	7.24	101.6	169.4	44.9	4.21,3.95	Y48	6.86	113.3	177.3	57.1	4.20
R19	8.39	120.4	174.4	54.7	5.63	K49	8.47	124.5	176.8	60.8	
V20	9.39	126.3	173.2	59.6	4.58	G50	9.52	116.7	173.7	44.9	4.80,4.41
S21	8.78	120.0	173.1	55.1	5.53	S51	8.41	116.3	171.1	58.7	4.63
F22	9.49	118.5	174.8	55.4	5.21	C52‡	9.96	114.4	175.6	55.6	5.94
E23	8.71	122.6	174.0	54.7	4.70	F53	8.68	122.6	174.4	57.7	4.87
L24	8.16	121.9	176.5	51.4	4.71	H54	7.49	118.8	174.8	57.0	4.66
F25‡	8.91	124.1	175.9	54.3	5.13	R55†	6.89	123.7	172.5	54.6	5.09
A26	8.47	128.5	175.6	54.1	3.75	I56†	9.15	126.7	173.1	61.2	4.60
D27	8.97	112.9	176.3	54.9	4.27	I57†	8.49	126.7		57.2	5.28
K28	7.54	117.4	176.2	56.2	4.56	P58	—		177.0	62.5	
V29	8.36	114.4		57.9	4.41	G59‡	9.83	113.7	171.5	44.8	4.04
P30	—		180.2	65.9		F60†	8.11	118.9	172.0	56.1	5.19

Table Ia

With the information obtained from selective labeling and the known primary sequence of CyP, the amides corresponding to the previously identified strings of adjacent amino acid residues were assigned. From the amide assignments, the carbonyl carbons were assigned by correlating the $^{1}H(\omega_3)$ and $^{15}N(\omega_1)$ amide chemical shifts of CyP to the carbonyl ^{13}C frequencies (ω_2) in a 3D HNCO [22] experiment.

The ^{1}H, ^{13}C and ^{15}N backbone assignments of CyP when bound to CsA are given in Table I. These backbone assignments are important for assigning the ^{1}H and ^{13}C resonances of the CyP side chains [29–31] and are critical for interpreting heteronuclear 3D [24] and 4D NOE [32–34] data sets used in the three-dimensional structure determination of the CyP/CsA complex (in progress). The current NMR data were analyzed in terms of the secondary structure of CyP when bound to CsA. Based on the NOE data obtained from the ^{15}N-resolved 3D NOESY-HSQC spectra, the secondary structure of CyP when bound to CsA was found to consist of an antiparallel β-sheet composed of 8 strands and 2 α-helices, similar to that determined previously by Wüthrich and coworkers [11] for uncomplexed CyP.

As indicated in Table I and Fig. 3C, several CyP amides shift upon binding CsA. These changes in chemical shift mainly occur in four regions of Cyp. These include CyP residues 55–74, 98–104, 112–116 and 120–126. Wüthrich and co-workers [10] reported similar shifts of CyP signals upon binding a water-soluble CsA derivative to CyP. Although these regions are far apart in the primary structure of CyP, the NOE data indicates that they are relatively close to one another in space (Fig. 4). On the basis of chemical shift differences between uncomplexed CyP and CyP bound to CsA or a

Res.	HN	^{15}N	C′	C^{α}	H^{α}
M61†	7.85	109.6	173.7	54.3	5.15
C62‡	8.32	112.6	172.2	57.3	4.81
Q63†	9.33	127.1	173.5	54.3	5.13
G64†	7.47	110.0	172.2	44.5	
G65‡	9.11	104.8	174.8	46.4	
D66‡	9.99	123.7	175.8	51.2	4.25
F67‡	6.73	115.5	173.5	55.8	4.61
T68	7.25	108.1	175.1	61.4	4.62
R69	8.70	121.5	175.6	54.6	4.38
H70†	6.58	110.7	173.6	56.8	4.42
N71‡	7.44	111.8	175.1	52.1	4.51
G72‡	9.66	110.7	174.5	44.8	4.47
T73†	7.87	109.2	175.6	61.9	4.15
G74‡	8.80	113.3	174.5	44.8	4.46,3.45
G75	8.09	108.5	171.5	42.9	4.57,2.64
K76	6.95	115.2	171.8	55.6	4.66
S77	7.79	114.1	175.1	56.7	5.21
I78	8.57	110.3	175.5	63.3	4.20
Y79	8.08	120.4	174.8	56.0	4.66
G80	7.12	105.9	173.4	42.7	4.65,3.80
E81	8.97	122.6	175.2	60.2	4.49
K82	7.89	112.2	174.8	53.5	5.54
F83	9.20	115.9	172.9	55.6	4.96
E84	9.25	118.9	175.6	56.2	3.78
D85	8.66	118.5	175.5	54.2	4.28
E86	9.56	131.5	175.6	60.0	
N87	7.07	106.3	170.0	52.4	4.12
F88‡	8.33	112.2	177.0	55.5	5.96
I89	8.31	119.3	177.0	64.6	3.74
L90	7.79	117.0	175.6	53.7	4.45
K91	8.09	118.5	175.6	54.1	4.60
H92†	10.58	121.9	177.0	56.3	4.34
T93	7.24	109.6	174.8	62.9	4.00
G94‡	7.54	106.6		45.3	4.75,4.44
P95	—		176.4	62.4	
G96‡	9.24	110.3	172.5	44.7	4.60,3.35
I97	6.78	120.7	172.1	59.2	4.02
L98†	7.82	129.3	173.0	53.0	4.81
S99‡	7.96	118.9	173.4	54.1	5.21
M100†	8.29	122.6	178.4	53.5	5.51
A101†	8.23	126.3	174.5	51.4	4.72
N102†	7.70	113.3	172.3	53.4	4.71
A103†	9.26	120.7	175.9	50.1	4.73
G104†	8.24	107.7		43.1	4.68,3.72
P105	—		175.5	63.7	
N106	8.86	118.9	174.4	54.0	4.11
T107‡	10.31	110.0	172.9	59.9	4.44
N108	7.43	120.0	173.4	55.5	4.20
G109	9.15	110.3	170.4	45.5	4.62
S110	8.77	116.7	175.3	57.1	4.78
Q111	8.42	124.5	175.5	57.9	4.29
F112†	8.17	117.0	171.4	55.0	6.05
F113†	9.83	114.4	172.1	54.7	5.85
I114†	9.03	117.0	176.7	58.7	4.86
C115†	9.50	124.1	175.9	61.1	4.58
T116‡	9.05	115.2	171.9	60.3	4.40
A117	7.62	121.9	174.5	50.4	4.37
K118	8.73	119.3	175.6	57.3	3.79
T119	7.38	117.4	174.5	56.7	3.55
E120†	9.09	124.1	176.6	59.0	5.19

Table Ib

water soluble CsA derivative [10], the CyP binding site for CsA can be inferred. However, changes in CyP chemical shifts may arise from either a direct interaction between CsA and CyP or a conformational change in the protein distant from the CsA binding site. Furthermore, the CyP chemical shift data cannot be used to determine the orientation of CsA in the CyP binding pocket. Further structural characterization of the CsA binding site was obtained from an analysis of CyP/CsA NOEs which were previously observed in ^{13}C-resolved 3D HMQC-NOESY spectra of uniformly ^{13}C-labeled CsA bound to CyP [9]. Using the NMR data obtained in this study, some of the previously observed intermolecular NOEs could now be assigned. These include: A101 (H^{α})/$Abu^{2}(H^{\gamma})$, A101(H^{β})/$Abu^{2}(H^{\gamma})$, A101(H^{α})/$MeVal^{11}(H^{\gamma 1})$, F60(H^{ε})/$MeVal^{11}(H^{\gamma 2})$, F60(H^{ζ})/$MeVal^{11}(H^{\gamma 2})$, F60(H^{δ})/$MeLeu^{9}(H^{\delta 2})$, and F60(H^{ε})/$MeLeu^{10}(NCH_3)$. As shown in Fig. 4, these NOEs together with CyP/CsA NOEs assigned previously between $MeLeu^{9}(H^{\delta})$ of CsA and the sidechain of W121 [35] can be used to orient CsA in the CyP binding site. Further characterization of the 3D structure of the CyP/CsA complex is in progress from an analysis of heteronuclear 3D [24] and 4D NOE [32–34] data which can be interpreted using the ^{1}H, ^{15}N, and ^{13}C assignments obtained in this study combined with those obtained from ^{13}C-^{13}C coherence transfer experiments [29–31].

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Res.	HN	^{15}N	C′	C^{α}	H^{α}
W121†	7.31	117.8	175.5	59.7	4.72
L122†	7.06	120.4	176.5	54.0	4.32
D123‡	7.59	121.9	177.5	55.4	5.18
G124‡	9.58	110.7	171.5	44.7	
K125†	7.59	114.1	174.4	55.9	4.06
H126‡	7.44	120.0	173.2	54.7	4.80
V127	8.25	124.1	174.1	63.4	4.24
V128	9.45	132.6	174.4	62.9	3.98
F129	8.12	116.7	172.1	55.6	5.24
G130	7.37	110.0	170.1	46.2	3.08
K131	8.30	114.4	174.4	54.5	5.16
V132	9.01	123.3	174.8	63.7	
K133	9.45	131.1	174.8	56.4	4.42
E134	7.54	117.7	174.3	55.0	4.55
G135	8.70	107.4	176.5	46.1	4.80,3.97
M136	8.88	122.9	177.0	56.5	4.46
N137	8.92	113.7	176.8	55.7	4.44
I138	7.65	123.7	177.0	61.1	3.75
V139	7.28	121.5	177.4	65.7	3.86
E140	8.31	117.4	178.7	58.9	4.67
A141	7.51	120.7	179.9	54.5	4.09
M142	8.31	116.6	177.2	59.3	3.96
E143	7.89	115.9	177.3	58.7	3.74
R144	7.01	114.1	176.6	57.3	3.92
F145	7.63	114.8	175.2	57.7	4.79
G146	7.57	104.4	171.2	43.7	4.59,3.61
S147	8.21	109.2	174.8	57.8	4.80
R148	8.93	120.0	176.2	59.4	
N149	7.87	110.7	176.2	52.1	4.83
G150	8.07	110.3	172.7	44.6	4.17,4.00
K151	7.56	119.3	178.0	56.8	4.40
T152	8.88	116.7	175.4	59.5	5.57
S153	9.47	117.4	174.0	58.7	4.42
K154	7.54	118.5	173.8	54.3	4.53
K155	8.80	121.1	175.5	56.2	4.30
I156	9.63	133.7	175.8	58.8	5.08
T157	9.23	117.0	173.0	59.1	5.30
I158	8.58	121.1	174.0	60.5	4.22
A159	8.86	131.9	177.9	53.9	4.12
D160	8.06	110.7	172.2	52.4	4.87
C161	8.59	115.5	171.9	55.0	4.53
G162	6.85	104.0	169.6	45.1	3.51
Q163	9.09	120.4	174.8	54.8	5.05
L164	8.59	125.6	175.5	54.5	4.62
E165	8.15	125.6		57.8	4.15

* ^{1}H and ^{13}C chemical shifts are reported relative to TSP. ^{15}N chemical shifts are referenced to $NH_4{}^{15}NO_3$ (376.25 ppm)[36].

† Amide cross-peaks observed in the HSQC spectrum of [U-^{15}N]CyP/CsA that do not match any of the cross-peaks in the HSQC spectrum of uncomplexed CyP within the range of ± 0.7 ppm for ^{15}N and/or ± 0.17 ppm for ^{1}H.

‡ As for (†) but within the range of ± 0.2–0.7 ppm for ^{15}N and/or ± 0.08–0.17 ppm for ^{1}H.

Table Ic

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