^{1}H NMR Studies on Bovine Cyclophilin: Preliminary Structural Characterization of Its Complex with Cyclosporin A†

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ABSTRACT: Cyclophilin (163 residues, M_r 17 737), a peptidyl prolyl cis–trans isomerase, is a cytosolic protein that specifically binds the potent immunosuppressant cyclosporin A (CsA). The native form of the major bovine thymus isoform has been analyzed by 2D NMR methods, COSY, HOHAHA, and NOESY, in aqueous media. The 156 main-chain amides in CyP yield 126 observable NH/αCH couplings (81%, Gly pairs counted as 1). Following exhaustive D_2O exchange, 44 amide resonances remain visible. Further analysis of the NH/NH, NH/αCH, and αCH/αCH regions of the COSY and NOESY data sets indicates that the residual amides in D_2O form a coherent hydrophobic domain which yields 2D NMR features suggestive of a β-sheet. Many (43/126) of the amide resonances have been classified according to amino acid type. In the aromatic region of the spectra, the assignment of the ring spin systems is nearly complete (12/15 Phe, 2/2 Tyr, 1/1 Trp, and 3/4 His). This has successfully lead to the complete assignment of all of their βCH's, main-chain αCH resonances, and many of the backbone amide resonances (8/12 Phe, 2/2 Tyr, 1/1 Trp, and 2/3 His). In other regions of the spectrum, the side-chain and main-chain resonances for 10/23 Gly, 9/9 Ala, 5/11 Thr, 5/9 Val, and 1/6 Leu have been completely assigned. The drug-free cyclophilin and CsA-bound cyclophilin form two discrete protein structures that are in slow exchange on the NMR time scale. Comparison of the fingerprint regions from the COSY spectra obtained from the two forms of the protein reveals a minimum of 16 cross-peaks which are clearly shifted upon complexation. In fact, on the basis of chemical shift changes observed in assigned side-chain and main-chain resonances, only a relatively few of the amino acid residues identified to date are perturbed by complex formation. These include 3 Phe (8, 12, and 14) and the Trp in the aromatic region and 2 Ala (7 and 8) in the Ala/Thr region. In the upfield-shifted methyl region, an assigned Leu and Val spin system and a spin system labeled X10 (an Ile or Leu) are affected by complex formation. In addition, a new aliphatic spin system, labeled X11, which shows a close spatial relationship to the perturbed Phe12, is observed in this region of the spectrum. In summary, the regions of the protein altered by complex formation can be divided into two categories: a hydrophobic and a H_2O-accessible domain. The hydrophobic region, defined in the D_2O exchange data sets, involves seven of the sixteen shifted NH/αCH COSY cross-peaks and includes Leu1, Val1, and Phe12. The other nine perturbed NH/αCH COSY cross-peaks occur in a region of the protein that is accessible to D_2O exchange in drug-free cyclophilin and includes the Trp, Ala7, Ala8, and X10 residues.

Cyclosporin A (CsA)[1] is a highly specific and potent immunosuppressant used clinically for the prevention of allograft rejection and treatment of graft versus host disease (Kahan, 1983). While CsA can affect a variety of biological events, particularly relevant being its effect on the early stages of helper T cell activation (Palacios, 1982; Hess & Tutschka, 1980) and the inhibition of interleukin 2 mRNA transcription (Kronke et al., 1984; Elliot et al., 1984), the molecular mechanism of action for CsA activity has yet to be determined.

[^{3}H]CsA has been shown to have a highly specific affinity for a low molecular weight cytosolic protein, termed cyclophilin (CyP), initially identified in BW5147 thymus cells (Merker & Handschumacher, 1984; Merker et al., 1983). This protein also demonstrated affinities for CsA analogues in proportion to their immunosuppressant activities (Handschumacher et al., 1984). CsA binding to cyclophilin has been shown to cause a 2-fold enhancement of the intrinsic fluorescence of its single Trp residue (Handschumacher et al., 1984). Studies of more than 100 CsA analogues indicate that immunosuppression required that the compound have an affinity for CyP; however, not all that bind possess immunosuppressant properties (Quesniaux et al., 1987). Such a correlation strongly supports the proposal that cyclophilin plays an integral role in the immunosuppressant activity of CsA. This proposal has been made even more intriguing by the recent finding (Takahashi et al., 1989; Fischer et al., 1989) that cyclophilin catalyzes the cis–trans isomerization of peptide bonds involving the prolyl residue, the rate-determining step for possible crucial protein-folding processes in the immune response.

Major and minor isoforms of cyclophilin have been detected in all normal and neoplastic mammalian cells tested (Koletsky et al., 1986), and the protein has been purified to homogeneity from human spleen and calf thymus cytosol (Harding et al., 1986). Major and minor isoforms of bovine (pI 9.4, 9.6) and

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[1] Abbreviations: NMR, nuclear magnetic resonance; CyP, cyclophilin; CsA, cyclosporin; COSY, correlated spectroscopy; HOHAHA, homonuclear Hartmann–Hahn; NOESY, nuclear Overhauser enhancement; TPPI, time-proportioned phase incrementation; BME, β-mercaptoethanol; DTT, dithiothreitol.

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human (p*I* 9.1, 7.4) CyP have been isolated and found to have similar molecular weights (17K) and to specifically bind one molecule of CsA ($K_d \approx 3 \times 10^{-8}$ M) (Harding et al., 1986). The complete amino acid sequence for the major isoform of bovine cyclophilin (163 residues, M_r 17 737) has been determined (Harding et al., 1986). The minor isoform of bovine CyP is sequentially identical with the major isoform but has an acetylated N terminus (unpublished data). Genetic studies indicate that the cDNA from a human leukemic cell line and rat brain have greater than 95% sequence identity with bovine cyclophilin (Haendler et al., 1987; Danielson et al., 1988).

Our primary objective was to elucidate the structural details of the CsA-binding site on cyclophilin. The strategy that we have adopted involves the use of ^{1}H nuclear magnetic resonance (NMR) methods for studies in the following distinct areas: (I) characterization of the free protein, CyP (Dalgarno et al., 1986); (II) identification of the unique spectral changes observed upon formation of the CsA–CyP complex; (III) the use of NMR-labeled CsA analogues to highlight specific conformational features and points of contact of CsA in the CyP binding pocket; and (IV) conformational analysis of the drug, CsA (S. L. Heald and I. M. Armitage, unpublished results). The results presented in this paper focus only on the first two areas and extend the earlier assignment of spin systems in the aromatic and upfield methyl regions of the ^{1}H NMR spectra in the drug-free protein (Dalgarno et al., 1986). While the NMR analysis is still in its infancy, these studies have revealed new information in the following areas: (1) progress toward the characterization of secondary structural elements of CyP, (2) spectral evidence for two distinct domains in CyP being affected by CsA binding, a hydrophobic domain and a H_2O-accessible domain, and (3) identification of several of the amino acids perturbed by CsA complexation.

Materials and Methods

Protein Preparation. The major isoform of calf thymus cyclophilin was purified to homogeneity as previously described (Harding et al., 1986) with the following improvements. The eluate from Matrex Blue A affinity chromatography was concentrated to 15–20 mg of protein/mL at 6 °C in an Amicon MC-10 ultrafiltration device with a YM-10 membrane. The major isoform was then isolated by preparative HPLC chromatography on a Waters ACCELL carboxymethyl cation-exchange resin (2.5 × 30 cm) with isocratic elution by 5 mM potassium phosphate (KP_i/45 mM NaCl/pH 6.9 at 6 mL/min). The salt concentration was increased to 200 mM, β-mercaptoethanol was added, and the pH was adjusted to 7.2 prior to storage at 4 °C. To obtain the ultrapure cyclophilin sample used in the phase-sensitive COSY experiment, the protein sample was further purified on an analytical TSK G/2000 size exclusion HPLC column (0.7 × 60 cm; 20 mM KP_i/200 mM NaCl/pH 6.8). For purposes of the NMR studies, the sample (20–40 mg) was concentrated by ultrafiltration to 5 mL and diafiltered into the desired buffer, generally 20 mM KP_i/200–300 mM NaCl/2 mM dithiothreitol (DTT)/pH 6.8. To minimize the adsorption of cyclophilin onto the YM-10 membrane used in the ultrafiltration device, the membranes were pretreated with a 2 mg/mL albumin solution and then washed with buffer prior to use. The final sample was further concentrated under N_2 to 0.4 mL, and 0.04 mL of D_2O was added before transfer to the NMR tube. The tube was flushed with argon and sealed with a cap and parafilm.

The CsA–CyP complex was generated from the NMR sample of purified cyclophilin by agitating the protein solution in the presence of 2 molar equiv of pulverized CsA at 6 °C for 4–6 h. Unsolubilized CsA was removed by centrifugation (14 000 rpm/20 min). The complex was transferred directly to an NMR tube and treated in the same manner as above.

NMR Methods. One- and two-dimensional NMR spectra were acquired on a Bruker AM500 NMR spectrometer. All data sets were processed on either a Sun 4/280S or a VAX 11/750 equipped with an array processor using the FT NMR software from Hare Research, Inc.[2] Correlated spectroscopy, COSY (Aue et al., 1976), experiments were obtained in the absolute-value mode, while double-quantum-filtered COSY (Rance et al., 1983), phase-sensitive COSY (Marion & Wüthrich, 1983), homonuclear Hartmann–Hahn, HOHAHA (Bax & Davis, 1985; Summers et al., 1986), and nuclear Overhauser enhancement, NOESY (Macura & Ernst, 1980) experiments employed time-proportional phase incrementation (TPPI) (Marion & Würthrich, 1983) in the absorption mode. The spectra consisted of 2048 complex data points in t_2 with some 400–530 t_1 experiments acquired. Phase cycling procedures enabled quadrature detection in t_1. All spectra were acquired with the carrier offset placed on the HOD resonance and the HOD signal attenuated by presaturation. Composite 90° pulses were used for the second and third pulses of the DQ filter in the phase-sensitive COSY sequence (Freeman et al., 1980). The HOHAHA experiments utilized mixing times of 18–43 ms during the MLEV-17 sequence, and one is reminded of the fact that these spectra sometimes suffer from cross-peak cancellation due to the competing buildup of the ROESY signal during the MLEV mixing period (Griesinger et al., 1988). The NOESY spectra were recorded with a mixing time of 175 ms, and this was randomly varied by 10% in order to remove coherent transfer effects (Macura et al., 1982). Water suppression and the base line were improved in the NOESY spectra by the use of a composite 90° pulse $(x, y, -x, -y)$ for the read pulse (Bax, 1985). The HOHAHA and NOESY data sets were processed by using a 30–45° shifted sine bell weighting function in both the t_2 and t_1 dimensions prior to Fourier transformation. Weak cross-peaks in the phase-sensitive COSY were significantly enhanced by strong right-handed skewed cosine apodization prior to Fourier transformation in the t_1 axis (Bax, 1982). Final matrices were 1K × 1K and have not been symmetrized.

In our first cyclophilin NMR publication (Dalgarno et al., 1986), unassigned aliphatic spin systems were labeled L1, L2, L3, etc. Significant progress has been made in the assignment of many of these resonances to amino acid types and their respective labeling with the conventional one-letter amino acid code. Consequently, we have now relabeled these resonances as X1, X2, X3, etc., in order to avoid confusion with their assignment to the amino acid leucine.

Results and Discussion

Drug-Free Cyclophilin. The acquisition of large quantities of protein required for these NMR studies was made possible through the development of improved methods for optimizing the yields and purity of CyP and enhancing its subsequent stability. The former was achieved through the integrated use of preparative cation-exchange HPLC and analytical size exclusion HPLC chromatography methods while the latter was accomplished by the addition of antioxidants (BME and DTT), high salt (>200 mM NaCl), low temperature for storage, and a pH in the range of 6.8–7.5.

The CyP used in our earlier NMR studies (Dalgarno et al., 1986) was exchanged with D_2O to facilitate these initial

[2] Hare Research, Inc., 14810 216th Ave. N.E., Woodinville, WA 98072.

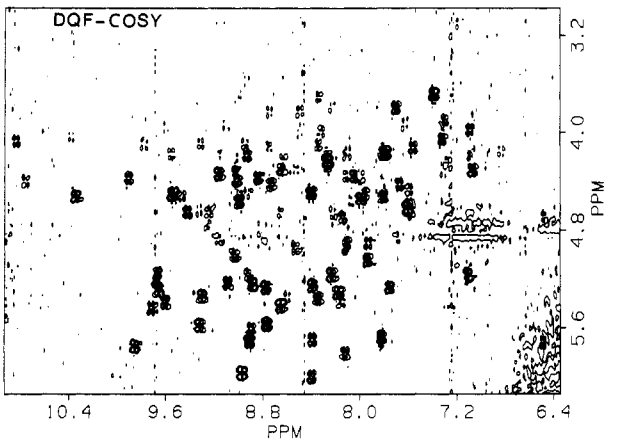

FIGURE 1: Expansion plot from the double-quantum-filtered COSY spectrum of nonexchanged drug-free cyclophilin (3.0 mM protein in 10% D_2O/10 mM KP_i/300 mM NaCl/10 mM DTT/pH 6.8; 25 °C; final purification achieved via size exclusion chromatography) showing the fingerprint region (w_1 = 3.09–5.88 ppm, w_2 = 6.37–10.67 ppm). Both positive (+) and negative (−) contour levels have been plotted: (+) in black and (−) in grey. For the double-quantum-filtered COSY, 512 t_1 experiments (each having 128 transients) were collected by using composite pulses for the second and third 90° pulses. The data were processed by using trapezoidal and right-skewed cosine apodization. Zero filling was added prior to Fourier transformation to yield a final matrix of 2K × 2K points.

measurements. All of the current NMR studies, however, employed a H_2O-buffered solvent system, thereby enabling us to begin assimilating spectral information regarding both the primary and secondary structure from assignment of the main-chain amide and αC proton cross-peaks. By use of a highly purified sample of cyclophilin in H_2O, 126 NH–αCH cross-peaks (Gly pairs counted as 1) were observed in the phase-sensitive COSY spectrum shown in Figure 1. Several of the weaker cross-peaks in this data set were confirmed from HOHAHA experiments. Of the 156 amide resonances in CyP (−7 for 6 Pro and the terminal NH_2), this represents 81% of the expected cross-peaks, a respectable number considering the rather narrow window found for this protein's stability and the fact that, at 17.7 kDa, CyP currently represents an upper limit in protein size manageable by conventional 2D NMR methods at 500 MHz. The success of these methods is attributed to at least three factors: (1) the use of a phase-sensitive COSY pulse sequence which incorporated composite pulses to sharpen the excitation profiles (Freeman et al., 1980); (2) optimized data processing in both t_1 and t_2 (Bax, 1982); and (3) more rigorous purification of CyP. Previous CyP samples purified by preparative cation-exchange chromatography alone and analyzed by conventional COSY and double-quantum-filtered COSY experiments gave only 50–60% of the expected cross-peaks in the fingerprint region.

Primary Structural Analysis. Significant progress (43/126; 34%) has been made in classifying the observed amides according to amino acid type, with particular attention being given to the hydrophobic residues which comprise about 50% of the protein. These results are summarized in Table I. For a protein of this size, NH–αCH cross-peaks involving complex multiplicities, e.g., Gly's, frequently yield weak or no cross-peaks in COSY data sets. This phenomenon was also observed in the NMR analysis of the ribosomal protein E-L30 by Van de Ven and Hilbers (1986). Of the 23 Gly residues in GyP, only 10 Gly NH resonances can be positively assigned even though 20/23 Gly main-chain geminal αCH pairs can tentatively be accounted for on the basis of their unique cross-peak shape in the HOHAHA, COSY, and NOESY spectra. None of the 10 assigned Gly NH resonances gave rise to 2 NH/αCH

Table I: Compilation of NMR-Detectable Amino Acids in Cyclophilin and Its Complex with Cyclosporin A

	CyP assigned to		CsA–CyP assigned to		
residue and no.	$C_\alpha H$	NH	$C_\alpha H$	NH	perturbed in CsA–CyP
hydrophobic (+CsA)					
Ala, 9 (2)	9	9	8	8	2
Gly, 23 (1)	20	10	5	5	2
Ile, 11	nd	nd	nd	nd	nd
(X10)	1	1	1	1	1
Leu, 6 (4)	1	1	1	1	1
Pro, 6	nd	nd	nd	nd	nd
Phe, 15	12	8	8	12	3
Val, 9 (2)	5	5	5	5	1
total = 79	47	33[a]	32	27	8
hydrophilic					
Thr, 11	9	5	9	4	0
His, 4	3	2	2	1	nd
Tyr, 2	2	2	2	2	0
Trp, 1	1	1	1	1	1
total = 18	15	10	14	8	1

summary:	main-chain NH	detected in COSY
	CyP, 156	126
	CsA–CyP, 160	104

[a] X10 not included.

cross-peaks in the phase-sensitive COSY spectrum. The strategy that was adopted to assign these resonances involved the integrated use of HOHAHA and NOESY data along with the phase-sensitive COSY spectrum. Each cross-peak in the fingerprint region of the DQF-COSY spectrum was carefully compared to NOESY cross-peaks in this region. Each COSY cross-peak associated with three strong NOESY cross-peaks was considered as a possible Gly NH resonance. If close examination of the HOHAHA spectrum showed two cross-peaks corresponding to a pair of αCH resonances of a Gly residue, then the Gly NH resonance was positively assigned. A complete list of all the resonance assignments in cyclophilin is provided in Table II.

The phase-sensitive COSY and HOHAHA spectra also showed a significant increase in the number of cross-peaks visible in the upfield methyl region of the spectrum shown in Figure 2. This region of the spectrum contains many of the resonances arising from the 11 Ile, 6 Leu, and 9 Val residues. Previously, eight resolved methyl resonances (X1–X9; X7 = Leu γCH) were identified in this region, of which three resonances, X1, X2, and X7, were assigned to the $CH(CH_3)_2$ side chain of a Leu residue (Dalgarno et al., 1986). The βCH resonances of this Leu residue have now been positively assigned from the HOHAHA spectrum at a remarkably high-field-shifted position of 0.15 and 0.22 ppm, which has enabled the assignment of the αCH and NH resonances in this Leu. This same Leu NH resonance is also found in the D_2O data sets, and its side chain has previously been shown to be in close spatial relationship to the aromatic rings of Phe1 and Phe9 (Dalgarno et al., 1986). The use of phase-sensitive 2D NMR experiments has revealed a second aliphatic spin system, labeled X10, overlapped with the X7(γCH) and βCH resonances. Within the X10 spin system, six of the seven resonances required for its assignment as a Leu or Ile residue have been identified, which include a βCH at 0.18 ppm as well as its main-chain αCH and NH resonances (Table II). Two of the coupled resonances in the X10 residue, highlighted by a double box in Figure 2, are closely associated with the aromatic ring protons of the single Trp residue.

Three of the methyl resonances (X4, X6, and X9) were previously associated with Val residues (Dalgarno et al., 1986).

Table II: Resonance Assignments in ppm for the Major Bovine Thymus Isoform of Cyclophilin in 10 mM KP_i, 200 mM NaCl, and 2 mM DTT, pH 6.8 at 25 °C

residue	label	NH	αCH	βCH	others
aliphatics					
Ala	A1	9.67	5.21	1.11	
	A2	9.30	4.09	1.49	
	A3	8.92	4.18	1.35	
	A4	8.80	4.85	1.37	
	A5	8.74	4.11	1.29	
	A6	8.49	3.83	1.55	
	A7	8.10	4.35	1.17	
	A8	7.66	4.42	1.48	
	A9	7.55	4.12	1.64	
Gly	G1	9.77	4.10, 3.85		
	G2	9.32	4.63, 3.36		
	G3	8.92	4.48, 3.57		
	G4	8.62	4.27, 3.47		
	G5	8.39	4.31, 3.99		
	G6	8.07	4.55, 4.15		
	G7	7.98	4.36, 3.59		
	G8	7.62	3.92, 3.53		
	G9	7.36	3.97, 3.58		
	G10	7.08	4.16, 3.98		
Leu	L1	7.92	5.04	0.22, 0.15	γCH 0.41; δCH_3 −0.11, −0.61
	(X10)	7.06	4.30	0.18	(1.20, 0.72, 0.42)[a]
Thr	T1	9.53	4.50	4.30	γCH_3 1.06
	T2	8.91	5.70	4.15	γCH_3 1.20
	T3	8.89	5.60	4.69	γCH_3 1.37
	T4	8.04	4.52	4.35	γCH_3 1.37
	T5	7.70	3.79	3.93	γCH_3 1.06
	T6		4.07	4.50	γCH_3 1.26
	T7		4.52	4.48	γCH_3 0.92
	T8		4.38	4.48	γCH_3 0.87
	T9		4.79	4.15	γCH_3 0.75
Val	V1	9.55	4.17	1.76	γCH_3 0.74, 0.09
	V2	8.98	4.56	1.84	γCH_3 0.67, 0.34
	V3	9.41	4.65	1.79	γCH_3 0.88, 0.53
	V4	9.09	3.93	1.97	γCH_3 1.06, 0.62
	V5	7.29	3.90	2.51	γCH_3 1.04, 0.83
aromatics					
His	H1	8.22	5.14	3.19, 3.09	δCH 8.10; εCH 7.32
	H2		3.98	3.25, 1.89	δCH 7.86; εCH 7.03
	H3	8.71	4.41	3.27, 3.27	δCH 7.75; εCH 6.75
	H4	8.65	4.65	3.34, 3.22	δCH 7.54; εCH 6.86
Phe	F1		5.28	2.48, 2.35	δCH 6.59; εCH 6.20; ζCH 6.70
	F2		5.36	3.25, 3.15	δCH 7.56; εCH 7.24; ζCH 6.25
	F3	6.48	4.69	3.16, 2.75	δCH 6.69; εCH 7.05; ζCH 7.14
	F4	8.09	4.92	2.91, 2.55	δCH 6.96; εCH 7.26; ζCH 7.45
	F5				δCH 6.83; εCH 6.24; ζCH 7.25
	F6	8.96	5.96	2.86, 2.69	δCH 6.79; εCH 7.02; ζCH 7.09
	F7		4.82	3.57, 2.94	δCH 7.54; εCH 7.40; ζCH 7.64
	F8	8.65	5.43	2.60, 2.01	δCH 7.66; εCH 7.62; ζCH 7.50
	F9				δCH 6.87; εCH 6.48; ζCH 5.72
	F10	8.11	4.96	3.15, 3.05	δCH 7.41; εCH 7.27; ζCH 7.38
	F11		4.40	3.00, 2.71	δCH 7.22; εCH 7.36; ζCH 7.45
	F12	9.84	5.74	2.89, 2.83	δCH 6.88; εCH 6.82; ζCH 6.95
	F13	8.92	5.15	3.30, 2.71	δCH 6.93; εCH 7.05; ζCH 7.08
	F14	10.00	5.95	3.61, 3.09	δCH 6.60; εCH 7.56; ζCH 7.25
	F15				(6.85, 6.88, 7.14, 7.17)[a]
Trp	W1	7.30	4.72	3.38, 3.42	δ_1CH 7.06; ζ_2CH 7.71; ηCH 7.38; ζ_3CH 7.16; ϵ_3CH 6.68; εNH 9.83
Tyr	Y1	8.10	4.75	2.36, 3.46	δCH 6.46; εCH 6.16
	Y2	8.39	6.00	2.58, 3.56	δCH 7.29; εCH 6.91

[a] Cross-peaks in parentheses are not assigned to specific residue protons.

Their main-chain amide resonances have now been assigned (V1 = X4; V2 = X6; V3 = X9) on the basis of a COSY coupling to the assigned αCH and a NOESY cross-peak to the βCH. In addition, two new Val residues (4 and 5) have been completely assigned from the DQF-COSY and HOHAHA spectra. Four of these five Val NH resonances are found to remain after exhaustive D_2O exchange.

The $CH–CH_3$ group in the Ala and Thr residues gives rise to a strong COSY cross-peaks in a distinct region of the spectrum: approximately the spectral cross section of 4.3 ppm for the CH and 1.3 ppm for the CH_3. Of the 9 Ala and 11 Thr, 19 strong $CH–CH_3$ cross-peaks were accounted for in the phase-sensitive COSY spectrum. The cross-peaks arising from the Ala residues were differentiated from Thr by the fact that the Ala $CH–CH_3$ cross-peaks were directly coupled to an NH resonance which showed a strong NOE back to the βCH_3 resonance. For the Thr residues, on the other hand, the methyl resonance in the $CH–CH_3$ cross-peaks showed a strong NOE to an αCH resonance which was directly coupled back to the βCH resonance in the $CH–CH_3$ cross-peak. This αCH resonance was directly coupled to its main-chain Thr NH resonance which showed a strong NOE back to the βCH

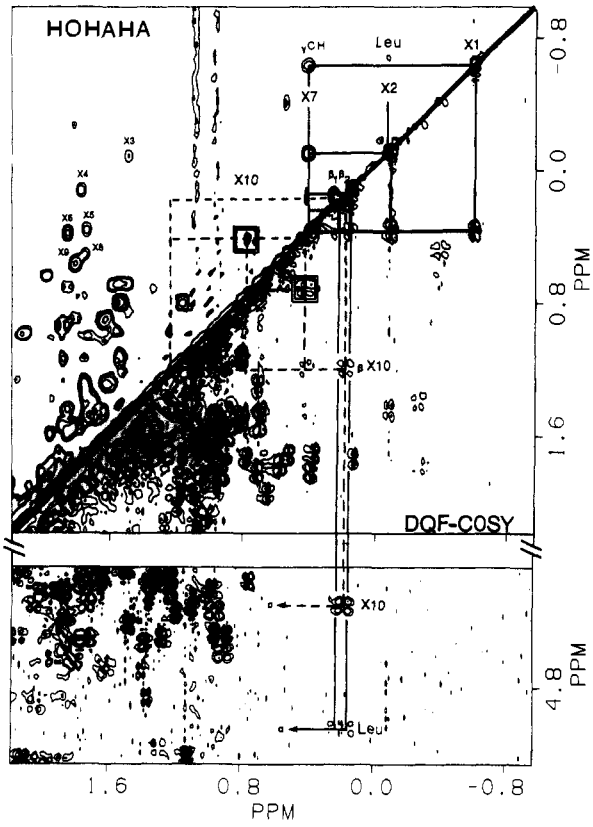

FIGURE 2: Composite of the expanded upfield methyl region from the HOHAHA spectrum (upper left) and the double-quantum-filtered COSY spectrum (lower right; spectral details given in Figure 1) of nonexchanged drug-free cyclophilin ($w_1 = w_2 = -0.97$ to 2.19 ppm). Below the composite spectrum is a portion of the upfield methyl/αCH region ($w_1 = 4.07$–5.26 ppm, $w_2 = -0.97$ to 2.19 ppm) taken from the double-quantum-filtered COSY spectrum. The leucine spin system has been connected by a solid line, while the X10 spin system has been connected by a dashed line. The scalar coupling cross-peaks which connect the two resonances in the X10 residue associated with the Trp ring NH by strong NOESY cross-peaks are double boxed. The cross-peaks corresponding to the original X1–X9 methyl resonances are labeled in the HOHAHA portion of the composite spectrum. The HOHAHA spectrum was collected on a 3.6 mM protein sample (final purification via weak cation-exchange chromatography) in 10% D_2O/10 mM KP_i/200 mM NaCl/2 mM DTT/pH 6.8; 25 °C). The HOHAHA spectrum was collected over 498 t_1 experiments of 128 scans each with a MLEV-17 mixing period of 23.8 ms and an effective field of 8333 Hz.

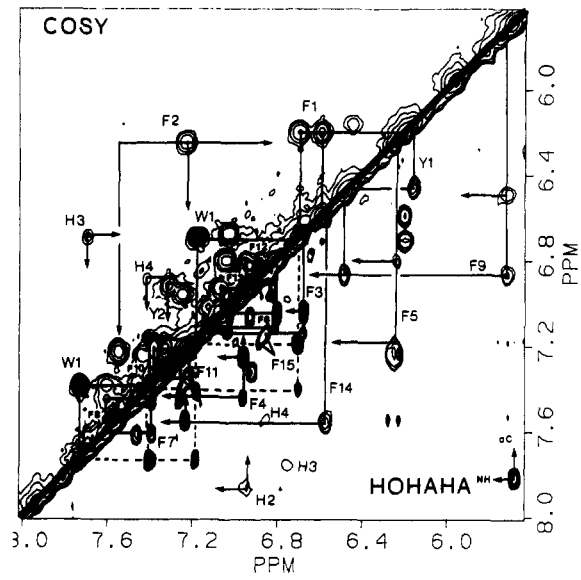

FIGURE 3: Composite of the expanded aromatic region from the COSY spectrum (upper left) and HOHAHA spectrum (lower right; spectral details given in Figure 2) of drug-free cyclophilin ($w_1 = w_2 = 5.62$–8.01 ppm). The COSY acquisition parameters have been published (Dalgarno et al., 1986). The COSY data were collected following exhaustive D_2O exchange on 1.7 mM protein in 20 mM KP_i/2 mM DTT/pH 7.95; 25 °C. The data were processed with unshifted sine bell apodization and zero filled to yield a 1K × 1K matrix. Labeling of the aromatic spin systems has been divided between the two spectra such that F1, F2, F8, F10, F12, F13, H3, and H4 are found in the COSY spectrum and F3–F7, F9, F11, F14, F15, and H2–H4 are found in the HOHAHA spectrum. Cross-peaks arising from the Trp spin system (W1) have been blackened and connected with dashed lines in both spectra.

resonance in the CH–CH_3 cross-peak. By use of these standard methods 9/9 Ala were assigned to their backbone NH resonances, while 9/11 Thr were assigned to their main-chain αCH and 5/11 to their main-chain NH resonances. The NH assignments in the Thr residues were partly dependent upon the observance of a NH/βCH NOESY cross-peak. In 4/11 Thr spin systems, this identification was hindered either by the second-order coupling of the αCH/βCH spins or by the occurrence of the αCH resonance next to the HOD signal. The missing cross-peak in this region is assumed to be due to a Thr residue.

The greatest progress in cataloging assignments of amino acid resonances has been made for the aromatic residues. The aromatic resonances in CyP arise from 15 Phe, 1 Trp, 2 Tyr, and 4 His, and representative 2D spectra of this region are shown in Figure 3 where the Trp spin system has been highlighted. Of these aromatic resonances, 6 Phe, 1 Trp, 1 Tyr, and 3 His were previously assigned from COSY, RELAY, and NOESY experiments in D_2O (Dalgarno et al., 1986). In this study, two new approaches have enabled the successful assignment of all of the aromatic ring spin systems with the exception of 1 Phe and 1 His. One involved the use of the unique set of resonances from the CsA-complexed form of the protein, and the other involved the use of the HOHAHA experiment which facilitated the identification of cross-peaks buried in the diagonal of the COSY spectrum. In addition, there are four Phe (5, 9, 14, and 15) which gave weak or no cross-peaks in the COSY spectrum but yielded weak cross-peaks in the HOHAHA spectra. These data may reflect the occurrence of a somewhat shorter T_2 relaxation time for these Phe resonances which would be better sustained in the HOHAHA spectra due to the decreased effect of T_2 decay during the HOHAHA mixing period (Bax & Davis, 1985). Shorter T_2 relaxation times could arise from the slower rotational motion associated with the clustering of phenyl rings which has been shown to result in stabilization of protein tertiary structure (Burley & Petsko, 1985). It would not be unexpected to find such an ordering of the phenyl rings in cyclophilin, considering their abundance.

The HOHAHA spectrum proved most useful in confirming the original assignment of the Trp ring spin system. The transfer of magnetization throughout the Trp ring protons, C_4H–C_7H, was seen to occur in the spectrum with mixing times of 41 ms. The Trp C_2H resonance was previously identified from spin-echo experiments (Dalgarno et al., 1986). With the assignment of the Trp nonexchangeable aromatic spins complete, the Trp ring NH can now be identified from the NOESY spectrum. In the NOESY data, one expects to see cross-peaks of approximately equal intensity from the Trp NH to both its C_2H and C_7H. By simply moving though the amide region of the NOESY data along the aromatic axis, only one NH resonance (9.83 ppm) is found which contains cross-peaks that match the C_2H and C_7H frequencies. This NH resonance shows the two strong NOEs to Trp C_2H and

C_7H, as well as an additional aromatic cross-peaks corresponding to Phe11 and two NOEs to a pair of coupled upfield methyl resonances that are assigned to the X10 residue.

Many of the main-chain αCH and NH resonances associated with these aromatic amino acid residues have now been assigned (see Table II) by first identifying the βCH resonances from NOESY cross-peaks to each assigned aromatic spin system. Since only four amide resonances occur in the aromatic region of the D_2O NOESY data set, 22 pairs of β-protons can be tentatively aligned with the aromatic spin systems (15 Phe, 2 Tyr, 1 Trp, and 4 His) in the βCH (3.6–2.2ppm)/aromatic cross section of the NOESY spectrum. The Trp βCH's were recognized from their NOEs to the Trp ring protons, C_2H and C_4H, and appear to be degenerative. In H_2O the Trp main-chain NH was also found in the aromatic region of the spectrum, and it showed a weak NOE to its β-protons. The assignments of the main-chain NH and αCH resonances in the Trp residue, as well as all of the other aromatic main-chain assignments reported herein, were confirmed by at least one observed βCH/αCH scalar coupling cross-peak and a corresponding αCH/NH cross-peak in the DQF-COSY and HOHAHA spectra. Further confirmation of these assignments came from the observation of both βCH's/αCH NOESY cross-peaks and at least one βCH/NH NOESY cross-peak. For both Tyr ring spin systems, the downfield aromatic doublet was found to be ortho ($C_{2,6}H$) to the side-chain protons. The main-chain αCH and NH for both Tyr in CyP was positively assigned as above.

Three of the four His were previously assigned based on spin-echo multiplet selection experiments and a pH titration over a limited range (Dalgarno et al., 1986). In CyP, each upfield spin showed a distinctive set of NOEs to βCH resonances in the D_2O data. These assignments were confirmed by comparing these cross-peaks to those observed in the H_2O NOESY spectrum, which was obtained at a different pH: 8.0 for D_2O and 6.8 for H_2O. The His ring protons characteristically shift downfield at lower pH. This results in the His C_2H/βCH NOESY cross-peaks also shifting downfield along the aromatic axis. This was especially useful in the case of His2 in which its C_2H overlapped with the Phe13 $C_{2,6}H$ resonance in D_2O but was resolved in H_2O, thereby allowing for the β-protons to be positively assigned for both aromatic residues. From the βCH assignments, main-chain αCH resonances were identified for all four His. Three His (1, 3, and 4) yield positive NH assignments, while that for His2 remains ambiguous.

The complex scalar coupling pattern for the phenyl ring enables the $C_{3,5}H$ resonance to be readily discernible. The assignments for the $C_{2,6}H$ and C_4H resonances were resolved through careful assessment of the remaining NOEs to β-protons in the aromatic region. This identified the side-chain resonances associated with each of the 14 assigned Phe rings and confirms the $C_{2,6}H$ resonance. (Note that the β-resonances for Phe5, -9, and -15 whose aromatic ring assignments are based on weak cross-peaks are not included in Table II, pending further investigation.) Main-chain αCH resonances were assigned from these β-protons. For 8/14 αCH directly coupled to NH resonances, the NH assignment was confirmed from observed NOESY cross-peaks back to the β-protons. However, the remaining six αCH resonances were all located near the HOD signal, and in every case, the assignment of the corresponding NH resonance could not be supported by reliable NH/βCH cross-peaks in the NOESY spectrum. All of the above assignments are listed in Table II.

Secondary Structural Analysis. As reported previously (Dalgarno et al., 1986), following exhaustive D_2O exchange, 44 cross-peaks are still visible in the fingerprint region of the COSY spectrum. These data, as well as the numerous interresidue NOEs observed between the side chains of the hydrophobic residues represented in the aromatic and upfield methyl regions of the NOESY spectrum, lead to the proposal of a compact hydrophobic cluster in CyP (Dalgarno et al., 1986). As seen in Table I, 33/126 amides have been assigned to hydrophobic residues, and 15 (4 Ala, 4 Val, 1 Leu, and 6 Phe) of these are retained in the D_2O data. Further analysis of the fingerprint, NH/NH, and αCH/αCH regions of the COSY and NOESY data sets indicates that many of these 44 residual amides form a coherent hydrophobic domain which yields 2D NMR features suggestive of a β-sheet. However, prior to the complete identification of the side-chain spin systems and the sequence-specific assignments of these main-chain amides, the model derived for the secondary structure of CyP from this NMR data must be viewed as tentative.

The approach we used to assess the D_2O data for secondary structural information most closely resembles the main-chain-directed (MCD) method proposed by Englander and Wand (1987) as an alternate approach to the sequence-specific assignment method described by Wüthrich et al. (1982). The MCD strategy is suitable for larger proteins, such as cyclophilin, in which the complete assignment of side-chain resonances, a requirement of the latter approach, is cumbersome. This method relies upon the recognition of NOESY cross-peak patterns involving the NH, αCH, and βCH resonances which characterize secondary structure and has been successfully applied in the analysis of ubiquitin (DiStefano & Wand, 1987). In this publication, we show the preliminary results from the interpretation of cross-peak patterns arising only from a tracing of the αCH and residual amide resonances in the D_2O spectra. While the neglect of the associated βCH resonances or the sequence-specific assignments of these residues may compromise the validity of our analysis, at this juncture, our intention is simply to illustrate the β-sheet secondary structure character reflected in some of the observed main-chain resonances in D_2O.

The short- and medium-range distances that uniquely characterize α-helix and parallel and antiparallel β-sheet have been comprehensively summarized by both Wüthrich (1986) and Wand (DiStefano & Wand, 1987). One distinguishing feature between an α-helix and a β-sheet is that the d_{NN} connectivities are signatures for an α-helix while the $d_{\alpha N}$ connectivities are characteristic of a β-sheet. The following describes the general features of the cross-peak patterns observed for cyclophilin in D_2O in the fingerprint, NH/NH, and αCH/αCH regions of the NOESY spectrum: (1) each of the resolved hydrophobic amide resonances in D_2O (33/44) shows a weak NOE to its own αCH and a strong NOE to another αCH resonance; 8/11 remaining amides also show a strong NOE to another αCH resonance, but their own αCH are unresolved; (2) only 12 weak NH/NH cross-peaks are observed involving less than half of the residual amide resonances; (3) 6 strong αCH/αCH cross-peaks are found in the low-field αCH region (4.8–6.0 ppm) (Wüthrich, 1986). Assuming the pattern in (1) above arises from $d_{\alpha N}$ connectivities in extended polypeptide chains, one can "walk" through 6–9 peptide strands comprised of 4–8 amino acids each. The uncertainity in this tentative sequencing of the cross-peaks prior to their sequential assignments arises from chemical shift degeneracies. However, the future inclusion of the βCH resonances and/or the se-

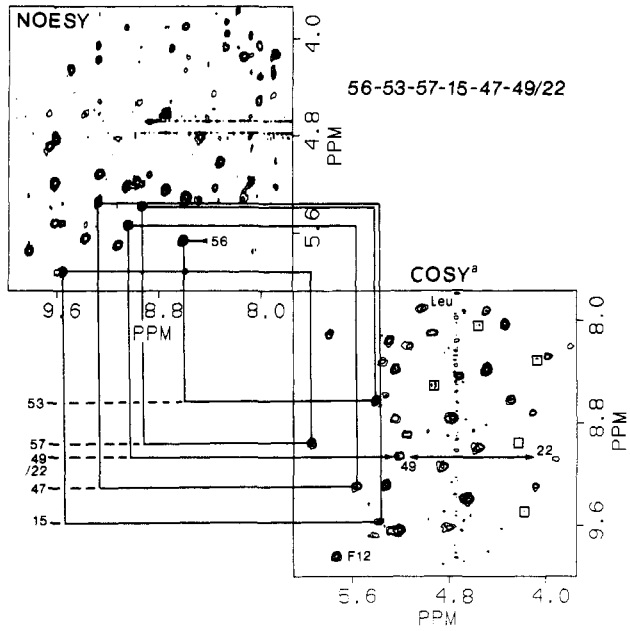

FIGURE 4: The sequential "walk" via $d_{\alpha N}$ connectivities begins with αCH resonance 56 in the phase-sensitive NOESY spectrum (upper left; w_1 = 3.73–6.06 ppm, w_2 = 7.76–10.00 ppm), moves in a counterclockwise pathway, and ends with the degeneracy of αCH resonances 22 and 49 in the COSY spectrum (lower right; spectral details provided in Figure 3, w_1, w_2 are reversed). The sequence 56–53–57–15–47–22/49 is shown. Details regarding the acquisition of the NOESY spectrum have been published (sample conditions are provided in Figure 3). This NOESY data set was collected with a mixing time of 100 ms and was processed with a 30° shifted sine bell in both dimensions (the first t_1 point was multipled by 0.4) and zero filled to yield a 1K × 1K matrix. For this figure only, the COSY spectrum was symmetrized. Of the 44 residual NH/αCH cross-peaks observed in the D_2O data sets, 40 can be counted in the COSY spectrum (weak cross-peaks have been boxed). Only the four NH/αCH COSY cross-peaks occurring in the aromatic region are not shown.

quence-specific assignments in a more rigorous application of the MCD strategy would eliminate much of this uncertainty.

One tentative pentapeptide sequence identified by the above procedures is illustrated in Figure 4, where the NH/αCH cross-peaks have been arbitrarily numbered for cataloging purposes. Using a combined COSY/NOESY spectrum, one can apply the technique adapted from Keller et al. (1983) and step along the peptide backbone from NH_i to αCH_i via a COSY cross-peak and then from the αCH_i to NH_{i+1} via a corresponding NOESY cross-peak. In the downfield portion of the fingerprint region shown in Figure 4, the "walk" begins with the NOESY cross-peak between the αCH, 56, at 5.70 ppm and the NH, 53. The 56 NH/αCH COSY cross-peak is not seen in D_2O due to the rapid exchange of this NH resonance indicative of its H_2O accessibility. From the 53 NH resonance, one moves through the 53 NH/αCH COSY cross-peak to the αCH resonance. The 53 αCH resonance cycles back to the NOESY spectrum and steps to the NH_{i+1} resonance, 57. One continues along the counterclockwise pathway indicated to establish the sequence: 56–53–57–15–47–22/49. The "walk" ends with a degeneracy for the αCH resonances, 22 and 49, observed in the COSY spectrum in Figure 4. There appear to be other possible degeneracies within this sequence; however, this pathway represents the "best fit" of the data at this time.

Even though the COSY/NOESY patterns for $d_{\alpha N}$ connectivities are implicit for extended polypeptide conformations, to justify secondary structure, such as β-sheet, one must demonstrate a neighboring β-strand sequence by extending the

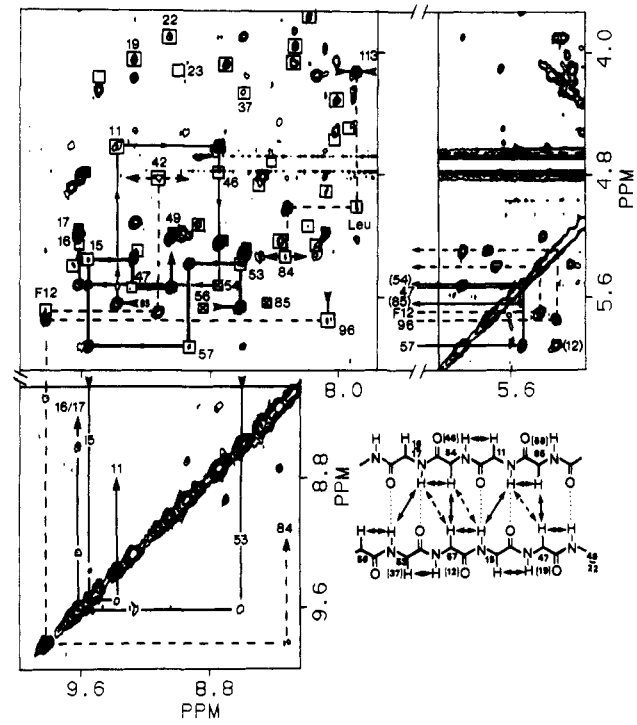

FIGURE 5: Three expanded sections from the NOESY spectrum described in Figure 4: portions of the fingerprint region, the αCH/αCH region (w_1 = 3.73–6.06 ppm, w_2 = 5.13–6.05 ppm), and the NH/NH region (w_1 = w_2 = 8.24–10.00 ppm). Within the expansion of the fingerprint region, the location of cross-peaks observed in the COSY spectrum (shown in Figure 4) is marked by boxes (black boxes are used in crowded regions of the spectrum). The sequential "walk" displayed in Figure 4 is accomplished here by first moving horizontally along a αCH_i resonance from the box indicating the COSY $\alpha CH_i/NH_i$ cross-peak to the NOESY $\alpha CH_i/NH_{i+1}$ cross-peak and then moving vertically along the NH_{i+1} resonance to the next box indicating the COSY $\alpha CH_{i+1}/NH_{i+1}$ cross-peak. Four sequential fragments are indicated in this figure: 56–53–57–15–47–49/22 is in a thick solid line; 85–11–54–16/17 is in a solid line; 23/113-Leu-84 and 96-F12-42 are in dashed lines. The NH/NH and αCH/αCH cross-peaks arising from the two "solid line" traced sequences are indicated with solid lines, and the NH/NH and αCH/αCH cross-peaks arising from the "dashed line" sequences are indicated by dashed lines. The cartoon drawing of two antiparallel β-stands represents the "solid line" sequence data only. NOESY cross-peaks connected by solid lines are indicated in the carton by solid arrows. NOESY cross-peaks which can be seen in the NOESY spectrum but have not been highlighted are indicated in the cartoon by dashed arrows. For each amino acid residue in the cartoon, any possible degeneracies with either the NH or αCH resonance are identified by the cataloged cross-peak number in brackets above the residue itself. The "dashed line" sequence fragments represent segments strongly perturbed in the CsA-complexed form of cyclophilin.

cross-peak pattern analysis to include main-chain NH/NH and αCH/αCH NOESY data. In the fingerprint region from the NOESY spectrum shown in Figure 5, the proposed sequence, 56–53–57–15–47–22/49, is represented by an alternate method, first used by Wagner (1986), in which the locations of all the COSY cross-peaks are indicated by boxes (black boxes in crowded regions). As can be seen, within the proposed sequence, NH resonances, 53 and 15, show NH/NH cross-peaks to resonances 16/17 and 11, respectively; while αCH resonances, 57 and 47, are involved in αCH/αCH cross-peaks to resonances 54 and 85, respectively. These NOEs are indicated by solid arrows in the cartoon of two antiparallel β-strands in Figure 5. Further inspection of the NOESY spectrum with regard to the main-chain resonances that bridge these two sequences reveals the additional NOE interactions which are indicated by broken arrows in the cartoon. Based on these NOEs, a second tetrapeptide sequence, involving

85–11–54–16/17, can be tentatively proposed. One can walk along the second β-strand starting with the 85 αCH and the αCH/αCH (47/85) cross-peak. 85 αCH gives a strong NOE to the NH_{i+1} resonance, 11. The 11 NH resonance shows NOEs across to 47 αCH and to 15 NH. The 11 αCH yield an NOE to the NH_{i+1} resonance, 54. The 54 αCH resonance gives NOEs across to 57 αCH (sequentially coupled to 15), to 15 NH, and also to the NH_{i+1} resonance degeneracy, 16 and 17. This NH resonance shows NOEs across to the 57 αCH and 53 NH (sequentially coupled to 57). This NOE pattern is completely consistent with that described for antiparallel β-sheet (DiStefano & Wand, 1987; Wüthrich, 1986).

It should be reemphasized that we have presented this example in its simplest interpretation without justifying all possible degeneracies with other NH and αCH resonances. These possible degeneracies are indicated by brackets in the cartoon in Figure 5. Our intention is not to sequence numerically cataloged NH/αCH COSY cross-peaks, but to illustrate the consistency of the NOESY and COSY data regarding the main-chain resonances in this example of the characteristic patterns for β-sheet. Moreover, many of the other residual amides and αCH resonances can readily be incorporated into additional cross-peak patterns which are consistent with parallel and antiparallel β-sheet. We believe at this time that a β-sheet secondary structure is present in the hydrophobic domain and that it is a mixture of parallel and antiparallel β-strands. In our ongoing investigations of the hydrophobic cluster, we are applying a more rigorous form of the MCD strategy which includes the βCH resonances, as well as continuing to assign side-chain resonances required by the sequential assignment approach.

Comparison of the NOESY spectra obtained in D_2O to that obtained in H_2O reveals noticeable increases in the number of symmetric NH/NH cross-peaks (12 in D_2O and >110 in H_2O). This is not too unexpected since NH/NH cross-peaks are expected for all the major forms of secondary structures in the following order of decreasing intensities: α-helix, β-turn, and β-sheet (Wüthrich, 1986).

CsA-Complexed Cyclophilin. Studies similar to those described above have been carried out on CsA-complexed cyclophilin. CsA forms a tight 1:1 complex with CyP which is in slow exchange on the NMR time scale. Very specific and reproducible changes occur in the protein ^{1}H NMR spectrum of the complex consistent with evidence for a specific binding site for CsA. In the following analysis, we have been able to extract information regarding several of the changes that occur in the protein by qualitatively comparing specific regions of the 2D NMR data sets obtained from free CyP to those data sets obtained on the CsA–CyP complex. Furthermore, in many instances resonance assignments could be extended by making use of the specific shifts induced in the CsA-complexed form of the protein.

CsA-complexed cyclophilin was generated by the microinfusion method, described under Materials and Methods. A comparison of the ^{1}H 1D NMR spectra at 500 MHz of the drug-free and the CsA-complexed forms of cyclophilin in an H_2O-buffered solvent system is shown in Figure 6. An expansion of the downfield-shifted NH resonances (10.3–12.6 ppm) is shown in inset A of Figure 6. Chemical shifts of this magnitude can arise from strong hydrogen bonding and/or anisotropic effects from aromatic rings. As can bee seen, two pronounced spectral differences occur in this region in the complex: a new NH resonance appears at 12.49 ppm, and several of the others are clearly shifted. Significant changes in the upfield methyl region are also observed, as shown in inset B of Figure 6. The 145-Hz chemical shift difference in the upfield methyl resonance enables one to put a limit on the off rate of CsA since this resonance can be shown in the following 2D NMR data sets on the complex to arise from the same methyl group. These changes have provided a very useful index for monitoring the formation of the complex.

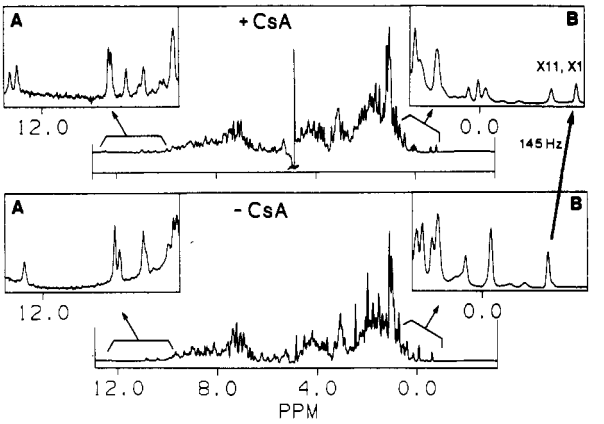

FIGURE 6: 500-MHz ^{1}H NMR spectra of 1.6 mM CsA-complexed cyclophilin (+CsA; top) and of 2.8 mM drug-free cyclophilin (-CsA; bottom). Both samples are in buffered H_2O (10% D_2O/10 mM KP_i/200 mM NaCl/pH 6.8; 25 °C; -3.2 to 13.0 ppm). The CsA-complexed form of cyclophilin was generated by a microinfusion method where excess cyclosporin A was removed by centrifugation. Water suppression was achieved by presaturation (period = 2.0 s). A spatially selective composite 90° pulse ($x, y, -x, -y$) replaced the read pulse to improve the base line. Insets A compare the resolved NH resonances at 10.7–12.4 ppm in the two forms of the protein, while insets B show the upfield methyl resonances at −1.0 to 0.7 ppm. The furthest upfield methyl resonance in drug-free cyclophilin has been reported as shifting 145 Hz upfield in CsA-complexed cyclophilin which 2D NMR data sets confirm arises from the same δ-methyl group in an assigned leucine residue; moreover, the X11 methyl resonance is unique to CsA-complexed cyclophilin and probably CsA-related.

We began our analysis of the 2D NMR data by comparing the fingerprint regions. Of the expected 160 (156 from CyP; 4 from CsA) amide resonances, 104 (65%) NH/αCH cross-peaks are observed in the double-quantum-filtered COSY and HOHAHA spectra. We might expect a significant improvement in this result through the use of an ultrapure sample of cyclophilin along with the improved phase-sensitive COSY pulse program which produced a 20–30% increase in observed NH/αCH cross-peaks in the drug-free CyP. Comparison of the fingerprint regions from the two forms of the protein reveals that 80 NH/αCH cross-peaks are virtually the same in both spectra. Of the remaining 46/126 cross-peaks in the spectrum of drug-free CyP, 16 strong cross-peaks are shifted upon complexation and the other 30 cross-peaks are ambiguous. In the spectrum of the complex, 8/30 cross-peaks are concealed by the HOD F_2 stripe, while the other 22 are weak cross-peaks in free CyP and so their absence in the spectrum of the complex is not surprising. In the DQF-COSY spectrum of the complex, 24/104 observed cross-peaks are clearly unique; 13 of these can be associated with the 16"perturbed" cross-peaks in drug-free CyP.

By comparing previously assigned side-chain spin systems in free CyP with data obtained under the same NMR conditions for CsA-complexed CyP, we are able to recognize discrete changes that occur in the protein upon complexation. A comparison of the aromatic region of the spectra is illustrated in Figure 7 in which the cross-peaks have been coded as follows: open = conserved; black = new or shifted, but assigned. As can be seen, 16 of 20 assigned spin systems appear to be the same in both spectra. The four aromatic spin systems which have been clearly shifted include the 1 Trp and

Table III: Resonance Assignments in ppm for the Cyclophilin–Cyclosporin A Complex in 10 mM KP_i, 200 mM NaCl, and 2 mM DTT, pH 6.8 at 25 °C

residue	label	NH	αCH	βCH	others
aliphatics					
Ala	A1	9.70	5.24	1.14	
	A2	9.27	4.09	1.52	
	A3	8.95	4.23	1.45	
	A4	(H_2O)			
	A5	8.78	4.15	1.33	
	A6	8.52	3.83	1.59	
	A7	8.16	4.30	1.23	
	A8	7.69	4.46	1.59	
	A9	7.58	4.16	1.68	
	A10		4.39	1.61	
	A11		4.73	1.22	
Gly	G4	8.63	4.29, 3.49		
	G5	8.42	4.33, 4.05		
	G6	8.10	4.56, 4.13		
	G7	8.00	4.38, 3.61		
	G9	7.40	4.02, 3.61		
Leu	L1	8.02	5.27	0.32, 0.23	γCH 0.16; δCH_3 0.00, −0.87
	(X10)	7.14	4.41	−0.08	(0.84, 0.58, 0.37)[a]
	(X11)				(1.49, 0.50, −0.64)[a]
Thr	T1	9.54	4.52	4.30	γCH_3 1.06
	T2	8.92	5.72	4.16	γCH_3 1.25
	T3	(H_2O)	5.65	4.72	γCH_3 1.37
	T4	8.08	4.38	4.55	γCH_3 1.41
	T5	7.73	3.83	3.93	γCH_3 1.06
	T6		4.07	4.52	γCH_3 1.31
	T7		4.52	4.48	γCH_3 0.92
	T8		4.38	4.48	γCH_3 0.87
	T9		4.79	4.16	γCH_3 0.77
Val	V1	9.70	4.10	1.75	γCH_3 0.82, 0.11
	V2	9.01	4.59	1.84	γCH_3 0.71, 0.39
	V3	9.44	4.67	1.81	γCH_3 0.92, 0.58
	V4	9.09	3.93	1.99	γCH_3 0.99, 0.61
	V5	7.33	3.93	2.53	γCH_3 1.09, 0.87
aromatics					
His	H1				
	H2		3.98	2.97	δCH 7.93; εCH 6.95
	H3	8.76	4.43	3.28	δCH 7.70; εCH 6.68
	H4				
Phe	F1		5.28	2.49, 2.35	δCH 6.58; εCH 6.20; ζCH 6.71
	F2		5.36	3.27, 3.42	δCH 7.55; εCH 7.23; ζCH 6.23
	F3	6.50	4.69	3.21, 2.79	δCH 6.71; εCH 7.06; ζCH 7.15
	F4	8.13	4.92	2.97, 2.45	δCH 6.94; εCH 7.25; ζCH 7.45
	F5				δCH 6.83; εCH 6.24; ζCH 7.23
	F6	8.98	5.98	2.91, 2.72	δCH 6.79; εCH 7.06; ζCH 7.15
	F7		4.82	3.63, 2.99	δCH 7.54; εCH 7.43; ζCH 7.64
	F8	8.64	5.42	2.58	(7.25, 7.33, 7.59, 7.78)[a]
	F9				δCH 6.90; εCH 6.48; ζCH 5.81
	F10	8.16	5.00	3.12, 3.08	δCH 7.41; εCH 7.27; ζCH 7.38
	F11		4.40	2.98	δCH 7.24; εCH 7.36; ζCH 7.45
	F12	9.84	5.94	2.59, 2.56	δCH 6.68; εCH 6.82; ζCH 7.34
	F13	8.96	5.12	3.33, 2.75	δCH 6.94; εCH 7.05; ζCH 7.08
	F14	10.13	6.01	3.63, 3.17	δCH 6.58; εCH 7.65; ζCH 7.42
	F15				(6.88, 6.90, 7.18, 7.21)[a]
Trp	W1	7.34	4.72	3.46, 3.48	δ_1CH 7.02; ζ_2CH 7.84; ηCH 7.55; ζ_3CH 7.43; ϵ_3CH 7.07; εNH 10.98
Tyr	Y1	8.10	4.75	2.42, 3.50	δCH 6.50; εCH 6.17
	Y2	8.39	6.03	2.54, 3.57	δCH 7.32; εCH 6.92

[a] Cross-peaks in parentheses are not assigned to specific residue protons.

3 Phe (8, 12, and 14) which have been highlighted in Figure 7. In many instances, the unique set of resonances from the CsA-complexed form of the protein proved very helpful for extending our resonance assignments. This is illustrated in the case of the previously assigned "R7" Phe, which was, in fact two overlapping phenyl rings, Phe7 and -8, in which Phe8 is shifted in the complex (Figure 7). A complete list of the assignments made in CsA-complexed CyP is provided in Table III.

The side-chain β-protons and main-chain αCH and NH resonances for the aromatic residues can be assigned by using the same methods described for the drug-free CyP. The β-protons yield essentially the same NOESY cross-peak pattern as observed for free CyP, with new βCH cross-peaks being identified for the shifted aromatic resonances of the Trp, Phe8, Phe12, and Phe14. The Trp βCH's are still degenerate and have only shifted about 0.1 ppm downfield. The strong NOEs observed between the Trp β-protons and the ring C_2H and C_4H protons has enabled a positive assignment of the Trp ring C_2H resonance. Positive identification of the Trp ring C_2H resonance allows the assignment of the Trp ring NH which is shifted 1.15 ppm downfield in the complex, from 9.83 to 10.98 ppm. In the NOESY spectrum, where the Trp ring NH showed two NOEs to the upfield methyl residue, X10, in drug-free CyP, six NOE cross-peaks can be found in the upfield region of the spectrum of the complex (vide infra, Figure

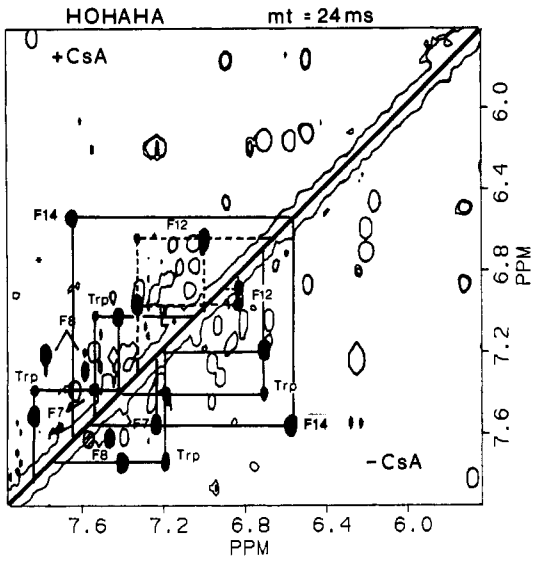

FIGURE 7: Composite of the expanded aromatic regions ($w_1 = w_2$ = 5.62–8.01 ppm) from the HOHAHA spectra obtained on CsA-complexed cyclophilin (+CsA; upper left; sample conditions are given in Figure 6) and on drug-free cyclophilin (–CsA; lower right; sample conditions and spectral details are given in Figure 2). Cross-peaks which are not affected by complex formation have been plotted with 2 contour levels and appear as open circles. Aromatic cross-peaks shifted in the CsA-complexed form of cyclophilin have been blackened. The Trp resonances are connected with solid lines, while F12 is highlighted with dashed lines. F8 and F14 are identified with labels. The HOHAHA spectrum on CsA-complexed cyclophilin was collected over 410 t_1 experiments of 176 scans each with a MLEV mixing period of 22.6 ms and an effective field of 8772 Hz. [(*) indicates that a cross-peak can be seen at a lower contour level.]

9). Three of these are related to the shifted X10 residue. The Trp backbone NH resonance, identified from its characteristic NOE to the β-protons, is shifted 0.1 ppm downfield from its position in drug-free CyP.

In addition to the large chemical shift changes observed for selected aromatic resonances, the Phe12 residue shows perturbations in the chemical shifts of its assigned βCH, αCH, and NH resonances. The Phe12 NH/αCH COSY cross-peak is one of the 13 identified cross-peaks that is clearly shifted in the complex. More importantly, this cross-peak is found in the D_2O data of the drug-free CyP and its neighboring (i + 1) and (i – 1) NH/αCH cross-peaks can be identified on the basis of the $d_{\alpha N}$ connectivity pattern described previously for the analysis of the fingerprint region observed in D_2O. This yields the following tripeptide sequence (highlighted by dashed lines in Figure 5): 96-Phe12-42, in which the neighboring residues have been identified by their catalogued cross-peak number (not amino acid sequence number). Furthermore, number 96 is also one of the 13 shifted cross-peaks in the complex, while number 42 is obscured by the F_2 HOD stripe. In both Phe12 and number 96, the αCH resonances show strong αCH/αCH NOESY cross-peaks to another residue, confirming that Phe12 and number 96 are both flanked by β-strands in an antiparallel β-sheet conformation. These two αCH/αCH cross-peaks are shifted in the NOESY spectrum of the CsA-complexed CyP. The β-protons in the numbers 96 and 42 amino acid residues can be identified from the D_2O data: 96 = 3.33 ppm and 42 = 2.11, 1.83 ppm. Though there are 15 phenylalanines in cyclophilin, all occur in unique tripeptide sequences. Therefore, identifying the neighboring β-resonances narrows down the number of sequence-specific assignments for Phe12 to the following four possibilities: ^{20}Ser-^{21}Phe-^{22}Glu, ^{34}Asn-^{35}Phe-^{36}Arg, ^{51}Cys-^{52}Phe-^{53}His, and ^{58}Gly-^{59}Phe-^{60}Met.

The Phe8 ring resonances are indicated with arrows in Figure 7 and in the complex show evidence for hindered rotation; i.e., four of the five spins can be assigned to the same Phe spin system. In completing the assignments for the Phe8 residue, the resonances for the ring spin system and β-protons are perturbed upon complexation, while the αCH and NH resonances remain unchanged. Therefore, only the side-chain protons of Phe8 are affected by complex formation. Both the Phe8 and Phe14 residues have been completely assigned and in both the amide resonances are located in the D_2O-accessible region of the protein.

The aromatic (labeled in Figure 7) and aliphatic resonances arising from the Phe14 residue are only slightly affected by complexation; however, one intriguing piece of evidence clearly implicates the Phe14 residue as being involved. The five furthest downfield amide resonances in the 1D spectrum of free cyclophilin (shown in inset A of Figure 6) yield unique NOE patterns which characterize their individual environments. On the basis of these cross-peaks, these five NH resonances can be reidentified in the same region of the NOESY spectrum of the CsA–CyP complex. The result of this inspection is that only the amide resonance in the 1D spectrum of the complex (see inset A of Figure 6) which is located the furthest downfield (12.49 ppm) is identified as being unique to the complex and possibly, therefore, CsA-related. This amide resonance yields a pair of NOESY cross-peaks to the aromatic resonances in Phe14 labeled in Figure 7.

Of the remaining aromatic residues, the two Tyr residues appear unchanged in the complex, while the information regarding the four His ring spin systems in the spectra of the complex is incomplete. Due to the small C_2H/C_4H coupling in the His ring spin system, only His2 and His3 yield cross-peaks in the COSY and HOHAHA spectra and are found to be unperturbed by complexation. All the data regarding the His4 residue are too weak to be reliable. An interesting finding, however, is that the C_4H side-chain and main-chain resonances tentatively assigned to the His1 residue are clearly shifted.

The aliphatic regions of the spectra have also been compared. A composite of the upfield methyl regions from the HOHAHA spectra of the drug-free and CsA-complexed forms of CyP is shown in Figure 8. A new aliphatic spin system, labeled X11, is evident in this region and is apparently unique to the spectra of CsA-complexed CyP; i.e., this resonance may arise from CsA. Only three resonances have been assigned to the X11 spin system: 1.49 and –0.64 ppm are scalar coupled, while 0.50 and –0.64 ppm show a strong dipolar coupling (Table III). In Figure 8, X1–X9 methyl resonances can be identified in the spectra of both forms of CyP. The Leu involving X1, X2, and X7 (γCH) is connected by solid lines, and it is the X1 methyl resonance that is positively identified as the methyl resonance that shifts 145 Hz upfield in the spectrum of the CsA complex. This Leu NH/αCH COSY cross-peak is one of the 13 cross-peaks that has been located in the spectra of both forms of CyP and is perturbed by complexation. This cross-peak is also found in the D_2O data and is involved in the following cross-peak sequence on the basis of the observed $d_{\alpha N}$ connectivity pattern (highlighted by dashed lines in Figure 5): 23/113-Leu-84. The neighboring (i + 1) NH/αCH cross-peak, 84, is coupled to a pair of βCH resonances at 3.22 and 2.84 ppm, while the (i – 1) neighbor is unresolved: cross-peak 23 or 113. The 84 NH/αCH COSY cross-peak is also identified and shifted in the spectra of the complex, and moreover, the NH resonance from 84 is dipolar

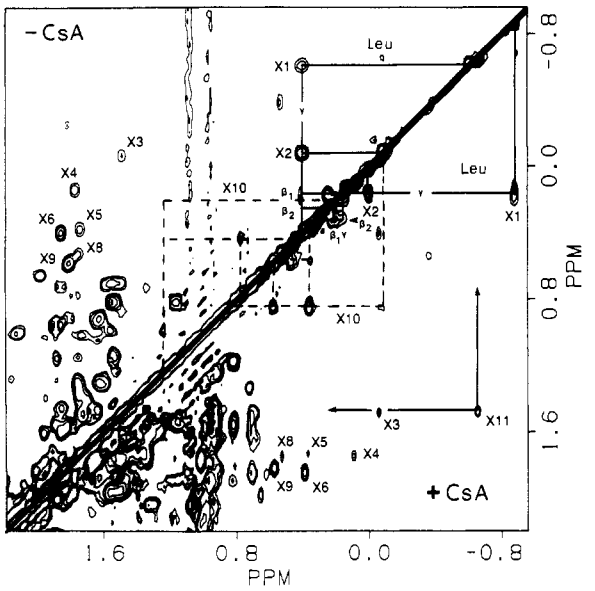

FIGURE 8: Composite of the expanded upfield methyl regions (w_1 = w_2 = −0.97 to 2.19 ppm) from the HOHAHA spectra of nonexchanged drug-free cyclophilin (−CsA; upper left; sample conditions and spectral details are provided in Figure 2) and CsA-complexed cyclophilin (+CsA; lower right; sample conditions are given in Figure 6; spectral details are in Figure 7). The aliphatic spin systems, Leu, X10, and X11, have been highlighted by connecting their cross-peaks. The previously cataloged upfield methyl resonances, X3–X6, X8, and X9, are unshifted in both spectra.

coupled to the NH resonance from the altered Phe12 (also in D_2O) by an observed NH/NH NOESY cross-peak. All six Leu residues in cyclophilin occur in unique tripeptide sequences. The identification of the βCH resonances in the (i + 1) neighboring residue narrows down the sequence-specific assignment to the following three possibilities: ^{22}Glu-^{23}Leu-^{24}Phe, ^{37}Ala-^{38}Leu-^{39}Ser, and ^{96}Ile-^{97}Leu-^{98}Ser.

For the remaining assigned spin systems in the upfield methyl region, the X10 residue, a Leu or Ile, is identified in Figure 8 and its main-chain αCH and NH resonances are assigned in the data on the complex. This residue is significantly perturbed upon complexation. Of the five assigned Val residues, four are unaltered by complexation. Val11, involving the X4 methyl resonance, is affected by complex formation. Both of the NH/αCH COSY cross-peaks assigned to X10 and Val11 are found to be shifted in the spectrum of the complex.

In the Ala/Thr region of the spectrum, six of the nine Ala are unchanged. This includes the observation of each NH/C_αH cross-peak in the DQF-COSY spectrum of the complex. Two of the $CHCH_3$ Ala cross-peaks, arising from 7 and 8, are clearly shifted. In the case of Ala7, the NH/αCH COSY cross-peaks is also perturbed, while the Ala8 NH/αCH cross-peak undergoes only a minor shift. Ala4 is concealed by the HOD signal in the spectra of the complex. For the Thr residues, four of the five Thr are unaffected by complexation, with their appropriate NH/αCH cross-peaks being identified in the DQF-COSY of the complex. The remaining Thr is hidden by the HOD signal. Two new Ala $CHCH_3$ cross-peaks are seen in data on the complex which can be tentatively assigned NH resonances from the list of 24 "new" NH/αCH cross-peaks observed in the complex. These Ala's are presumed to arise from CsA.

For the Gly residues, only five of the ten assigned Gly NH resonances are positively identified in the HOHAHA spectrum of the complex and are unaltered by complexation. Another one of the ten Gly NH resonances, assigned G1 in drug-free CyP, is clearly missing in the complex. The remaining four Gly NH resonances are ambiguous.

Of the 43 assigned amide resonances in drug-free CyP, 35 are present in the complex. The missing 8 are related to weak cross-peaks not seen in the DQF-COSY of the complex. Overall, 34% (35/104) of the NH/C_αH cross-peaks observed in the DQF-COSY spectrum of the complex have now been assigned. A complete list of all assignments made in the complex is provided in Table III.

Comparison of the aromatic–aromatic and aromatic–methyl regions of the NOESY spectrum obtained on the drug-free and CsA-complexed forms of CyP indicates the presence of a compact hydrophobic cluster in both forms of the protein which is found to be perturbed upon complexation. As seen in the HOHAHA spectra in Figures 7 and 8, most of the cross-peaks are conserved, which allows us to focus on the unique changes that occur upon complexation. These changes are best illustrated in the cross sections of the aromatic and upfield methyl regions in the NOESY spectra shown in Figure 9. The Trp ring C_2H and NH resonances show a close spatial relationship with a pair of resonances in X10 in the drug-free CyP. At this time, there is weak NOE evidence that F12 is also near to the Trp and X10 residues. The F12 ring resonances in drug-free CyP are closely packed together and occur in the most crowded portion of the aromatic region (see Figure 3). In the spectrum of the CsA–CyP complex, the NOE pattern associated with the Trp ring NH and C_2H resonances are distinctly different. Three NOE cross-peaks are now seen to the X10 spin system, as well as two additional aliphatic resonances at 0.9–1.0 ppm. In the upfield methyl region (not shown), the X10 resonance at 0.58 pm yields a distinct NOE to the X11 resonance at −0.64 ppm. As earlier noted, the X11 spin system has had three resonances (1.49, 0.50, and −0.64 ppm) assigned to date and apparently arises from CsA. The X11 spin system shows a strong set of NOE cross-peaks to the perturbed Phe12 ring. In addition, there now appears to be more cross-peaks in common to the Trp ring NH and Phe12. The conserved NOE patterns for the X1–X9 resonances allow for their ready identification between the two spectra.

Overall, the regions of the protein altered by complexation can be divided into two categories: hydrophobic and H_2O accessible. The hydrophobic domain, defined in the D_2O exchange data sets, involves seven of the sixteen perturbed amides sited in drug-free CyP, including 1 Leu, Val11, and Phe12. Of the remaining nine, six have been assigned to the Trp, Phe8, Phe14, X10, Ala7, and Ala8 residues, which are located in a region of the protein that is accessible to D_2O exchange. There is not enough evidence at this time to definitely assign either of these regions to the CsA-binding site and/or the site of biological activity. Unambigious assignment of resonances arising from CsA and the identification of NOESY cross-peaks reflecting CsA–CyP contact points would resolve this issue.

Conclusions

The 2D NMR structural elucidation studies of cyclophilin (CyP) and the CsA–CyP complex were limited by several anticipated problems. The protein is of moderate size, 17737 daltons, which results in relatively long correlation time and short T_2 recovery time. These effects were most apparent in the first COSY spectrum obtained on cyclophilin in which less than 50% of the NH/αCH cross-peaks were visible in the fingerprint region. Fortunately, we were able to increase this number to greater than 80% by careful optimization of the following three parameters: (1) data acquisition, (2) data processing, and (3) sample purity and stability. The restric-

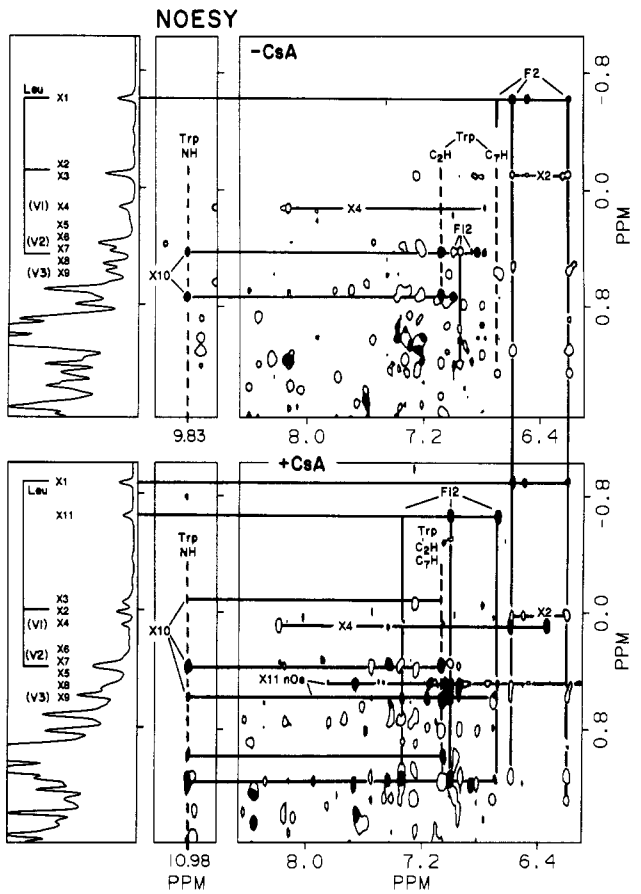

FIGURE 9: Composite of the phase-sensitive NOESY spectra obtained on drug-free cyclophilin (−CsA; top spectra; 2.3 mM protein in 10% D_2O/10 mM KP_i/2 mM DTT) and on CsA-complexed cyclophilin (+CsA; lower spectra; sample conditions given in Figure 6). The upper and lower parts of the figure consist of three panels each (left to right): 1D NMR spectrum of the upfield methyl region (w_1 = −0.97 to 1.41 ppm), NOESY cross section showing Trp ring NH (w_2 = 9.80–11.00 ppm) versus the upfield methyl regions, and NOESY cross section showing the aromatic (w_2 = 6.09–8.48 ppm) versus the upfield methyl region. Cross-peaks have been plotted with two contour levels only so the cross-peaks that are apparently the same in both spectra appear as open circles, while cross-peaks that are unique to each spectra have been blackened. Within the 1D NMR spectrum, the X1–X10 (X11 in "+CsA" only) resonances, as well as assigned Leu and Val residues, have been labeled. The NOESY cross-peaks identifying the association between the X11 residue and the F12 phenylalanine have been connected with solid black lines. The chemical shift position of the Trp ring NH and C_2H resonances and their NOESY cross-peaks and their NOESY cross-peak to the X10 residue are highlighted with dashed lines. The NOESY spectrum on drug-free cyclophilin was collected with a mixing time of 175 ms over 522 t_1 experiments of 160 scans each. Water suppression was achieved by presaturation (period = 1.2 s) and the use of a composite 90° pulse ($x, y, -x, -y$) for the read pulse. The data were processed with a 30° shifted sine bell in both dimensions (the first t_1 point was multiplied by 0.4) and zero filled to yield a 1K × 1K matrix. The NOESY spectrum on CsA-complexed cyclophilin was collected with a mixing time of 175 ms over 312 t_1 experiments of 128 scans each. The same water suppression and data processing methods as given above were used on this data set.

tions imposed by the protein's size were manifested in two facets of the 2D NMR spectra: poor resolution in certain regions of the spectrum and the loss of cross-peaks, particularly those involving complex couplings such as ABX spin systems. These limitations were partially overcome by the following two approaches: (1) the integrated use of COSY, HOHAHA, and NOESY spectra and (2) utilization of comparative data sets obtained in the two distinct forms of the cyclophilin, drug free and CsA complexed. Using these methods, we have been able to assign 30+% of the observed amide resonances according to amino acid type in both forms of CyP.

Our primary objective was to elucidate the structural details of the CsA–CyP interaction. Qualitative comparison of the data sets obtained on the drug-free and CsA-complexed forms of the protein reveals that less than 20% of the proton resonances in cyclophilin are affected by complex formation. In order to extract more structural detail regarding the CsA receptor site, 2D NMR data sets were analyzed by two parallel assignment strategies: (1) the sequence-specific method (Wüthrich et al., 1982) and (2) the main-chain-directed strategy (Englander & Wand, 1987). Using the former approach, we have identified 9 amino acids (2 Ala, 1 His, 1 Leu, X10, 3 Phe, 1 Trp, and 1 Val) that are perturbed by complexation. By comparing data sets on drug-free CyP in H_2O and following exhaustive D_2O exchange, we can subdivide these amino acids into two domains: hydrophobic and H_2O accessible. The sequence-specific strategy is somewhat limited at this point of our analysis, since it was never intended to deal with a selective region of the protein structure, i.e., the CsA-binding pocket, in a protein of this size.

Complexation with CsA, a lipophilic drug whose conformation in $CDCl_3$ was described by Kessler (Kessler et al., 1985) as a twisted β-sheet, imposes a change in the hydrophobic or non-H_2O-accessible region of the protein. In order to extract secondary structural elements in the hydrophobic domain prior to completion of the sequential amino acid assignments, the main-chain-directed strategy was utilized to evaluate the conformational properties reflected in the residual amide and corresponding αCH resonances. The cross-peak patterns assigned to this domain in the D_2O data sets appear to be consistent with a segment of β-sheet conformation. In fact, the data support the presence of a mixture of parallel and antiparallel β-strands. The 1 Phe and 1 Leu affected most by complex formation are located in this hydrophobic domain and involved in this antiparallel β-sheet conformation. These results suggest that CsA binds in the H_2O-accessible region of CyP and this, in turn, induces a change in the hydrophobic domain, or that CsA intercalates into the core of CyP, possibly forming a sheet-to-sheet association.

A final strategy utilized in resolving the CsA-binding pocket in CyP involves assigning resonances unique to CsA in the spectra of the CsA–CyP complex. We have identified 2 Ala and an aliphatic residue, X11, which appear to be unique to the spectra of the complex. The NOESY spectra have been searched for cross-peaks arising from the *N*-methyl resonances and the unique Bmt and Abu amino acid residues of CsA. These assignments have been more difficult than anticipated. We are presently using NMR-labeled CsA analogues to resolve specific sites in CsA and isotope-directed NOE studies to establish point of contact with CyP.

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We are greatly indebted to Paul R. Gooley for his acquisition of the spectrum shown in Figure 1 with the use of composite pulses in the double-quantum-filtered COSY experiment and for his inventive use of strongly skewed apodization; but mostly we enjoyed his enthusiastic insights into protein NMR research which were presented in his unique style of Australian humor.

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