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^{1}H NMR Studies on Bovine Cyclophilin: Preliminary Structural Characterization of This Specific Cyclosporin A Binding Protein†

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ABSTRACT: High-field ^{1}H NMR spectroscopy has been used to study the conformation of the cytosolic cyclosporin A binding protein cyclophilin. For the drug-free form of cyclophilin, spectral editing methods in conjunction with a pH titration were used to identify all four His residues present in the protein, and two-dimensional COSY and RELAY spectroscopy was used to elucidate the scalar connectivities in the aromatic and upfield methyl regions of the spectrum. From these scalar connectivities, it was possible to distinguish between inter- and intraresidue dipolar interactions within the aromatic and upfield methyl regions of cyclophilin in the NOESY spectrum. The results of this analysis showed extensive interresidue cross-relaxation among and between these latter spectral regions indicative of the proximal relationships of several of these residues and the presence of a hydrophobic core within cyclophilin.

Cyclophilin is the specific cytosolic binding protein responsible for the concentration of the immunosuppressant cyclosporin A (CsA) by lymphoid and nonlymphoid mammalian cells (Merker & Handschumacher, 1984). Furthermore, cyclophilin binds a series of cyclosporin analogues in proportion to their immunosuppressive activity in a mixed lymphocyte reaction, consistent with the hypothesis that cyclophilin plays an important role in the immunosuppressive action of CsA (Handschumacher et al., 1984).

Cyclophilin has been purified to homogeneity from bovine thymus and human spleen tissues. Two isoforms were identified from both of these sources that bind one molecule of CsA, have an apparent molecular weight of 17 kDa, and possess very similar amino acid compositions. The complete 163 amino acid sequence of bovine cyclophilin has recently been determined (M_r 17 737 Da) and found to contain a single Trp and four Cys residues. The first 72 NH_2-terminal residues of human cyclophilin were also determined and found to be identical with bovine cyclophilin, indicating a highly conserved structure. No significant sequence homologies with other protein sequences in the National Biomedical Research Foundation data base were detected (Harding et al., 1986). Additional physicochemical studies have shown that the CsA-binding activity of cyclophilin is sulfhydryl dependent and that the protein shows a 2-fold enhancement in the intrinsic fluorescence of its single Trp residue consequent to the binding of CsA (Handschumacher et al., 1984). These characteristics suggest that the Trp residue and probably a single Cys residue are critical determinants in the CsA binding site.

Our long-term objective is to use ^{1}H nuclear magnetic resonance (NMR) methods to elucidate the molecular details of the interaction between CsA and cyclophilin. Ultimately, this necessitates identification in the ^{1}H NMR spectrum of the amino acid residues involved in the CsA binding site and, subsequently, characterization of any protein conformational changes induced by drug binding. In this study, we present initial results in which both one- and two-dimensional ^{1}H NMR methods have been used to identify several of the spin systems in the ^{1}H NMR spectrum of the major isoform of bovine cyclophilin. These data were subsequently used to identify several spin systems coupled by interresidue cross-relaxation pathways in the NOESY spectrum. The identity and extent of the interresidue distance constraints derived from these data provide evidence for the the existence of a very hydrophobic core within the protein of suggested importance in the interaction with CsA.

Materials and Methods

Protein Preparation. The major isoform of calf thymus cyclophilin was purified to homogeneity as described previously

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(Harding et al., 1986) and stored at 4 °C as a dilute solution in the presence of 5 mM β-mercaptoethanol at pH 7.4 in 20 mM phosphate buffer prior to the preparation of the NMR samples. Once sufficient material had been accumulated for the NMR studies (5–15 mg), the samples were concentrated to ~1 mL at 6 °C in a Amicon MC-10 ultrafiltration device fitted with a YM-10 membrane. The protein was diafiltered into the desired D_2O buffer solution in the same device and concentrated down to 0.4 mL before transfer to the NMR tube. The tube was then sealed with a septum cap and degassed with argon. Final buffer conditions were 20 mM potassium phosphate and 1–2 mM dithiothreitol (DTT) (equimolar with the protein concentration), pH 8.0, with D_2O as solvent. This procedure was necessary to avoid lyophilization of cyclophilin, which results in an irreversible loss of CsA binding activity.

NMR Methods. One-dimensional spectra were acquired on either a Bruker WM500 or the Yale 490-MHz NMR spectrometer. Multiplet selection spectra were obtained by addition or subtraction of spin–echo and Carr–Purcell–Meiboom–Gill (CPMG) spectra, with the same total delay time ($1/J$) in each case (Campbell et al., 1975). With a total delay of 125 ms (J = 8 Hz), the spin–echo spectrum of the aromatic region contains singlet and triplet resonances in phase, with doublet resonances 180° out of phase (inverted), whereas in the CPMG spectrum all aromatic resonances are in phase. Addition of the two spectra results in cancellation of the doublet signals (singlet/triplet spectrum), and subtraction results in cancellation of the singlet and triplet signals (doublet spectrum).

Two-dimensional spectra were recorded on the Yale 490-MHz spectrometer and the data sets transferred to a VAX 11/750 computer and processed by the FTNMR software of Dr. Denis Hare. Correlated spectroscopy, COSY (Aue et al., 1976), and relayed coherence transfer spectroscopy, RELAY (Eich et al., 1982; Bax & Drobny, 1985), experiments were run in absolute-value mode, whereas the nuclear Overhauser enhancement spectroscopy (NOESY) experiments were run in absorption mode (Macura & Ernst, 1980; States et al., 1982). In each case, phase-cycling procedures permitted quadrature detection in t_1 and, therefore, the offset was chosen to lie at the HDO resonance position. NOESY experiments were recorded with mixing times of 50, 100, and 250 ms, and the mixing time was randomly varied by a few percent in order to remove coherent transfer effects (Macura et al., 1982). Mixing times for the RELAY experiments were calculated by the expressions of Bax and Drobny (1985), assuming a protein T_2 value of 0.03 s ($\Delta\nu_{1/2}$ = 10.6 Hz). Two RELAY data sets were acquired, one with a mixing time of 38 ms, optimized for aromatic ring system RELAY cross peaks, and the other with a mixing time of 25 ms, optimized for methyl/methylene/methine RELAY cross peaks. All data sets were symmetrized after processing (Baumann et al., 1981).

For the analysis of the ^{1}H NMR spectrum of cyclophilin, the following labeling scheme was adopted: aliphatic resonances are labeled L1, L2, L3, etc., with each label associated with a particular resonance; aromatic resonances are labeled R1A, R1B, R1C, R2A, etc., where R# denotes a particular aromatic spin system and A, B, C, etc., denotes the resonances that comprise this spin system. His resonances are labeled H1, H2, etc.

Results

The 500-MHz ^{1}H NMR spectrum of cyclophilin dissolved in D_2O is shown in Figure 1B. The spectrum is clearly indicative of a protein with a well-defined fold, with resonance

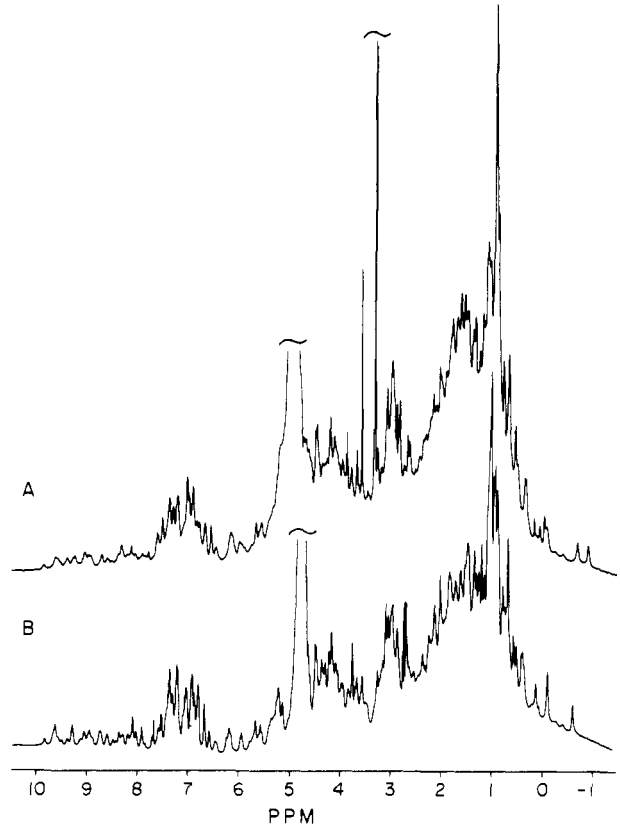

FIGURE 1: (A) 500-MHz ^{1}H NMR spectrum of cyclophilin, 0.2 mM in 20 mM potassium phosphate buffer, pH 7.95, in D_2O, after addition of 5 mol equiv of cyclosporin A from a concentrated stock solution in MeOH-d_4 directly to the NMR tube. Since cyclosporin A has low solubility in H_2O, this procedure resulted in considerable precipitation of cyclosporin, and thus the cyclophilin spectrum in Figure 1A is only approximately 90% saturated with cyclosporin A. Conditions: 0.25-s water presaturation pulse, 1024 scans with 70° flip angle, recycle delay 0.93 s, temperature 25 °C. (B) 490-MHz ^{1}H NMR spectrum of cyclophilin in the absence of cyclosporin A. Cyclophilin, 1.7 mM in 20 mM potassium phosphate, pH 7.95 in D_2O. Conditions: no water presaturation, 256 scans with 70° flip angle, recycle delay 0.68 s, temperature 25 °C.

line widths typical of those anticipated for a protein of this molecular weight at ambient temperature. Several noteworthy features can be identified in this spectrum. First, there are a considerable number of persistent NH resonances (approximately 40 appear in the COSY data set described later) arising from backbone amide protons that resonate downfield of the aromatic resonances, between 8 and 10 ppm. The intensity of these resonances is only slightly diminished after several weeks at neutral pH in D_2O buffer, indicating that regions of the protein backbone exhibit extremely slow amide-exchange rates perhaps as a result of their inaccessibility to the solvent. Additionally, in the high-field region of the spectrum (−1.0 to +0.6 ppm), several resonances have experienced large upfield shifts, which we will show later is due to ring–current shifts arising from their close association with aromatic residues. Finally, the well-dispersed aromatic region of the spectrum (6–8.2 ppm) suggests that a number of protein aromatic residues are also experiencing aromatic–aromatic interactions. Both of these latter two features suggest that a clustering of hydrophobic residues occurs in the protein's tertiary structure, which may constitute part of the binding site for the hydrophobic, cyclic undecapeptide cyclosporin A (CsA).

Changes induced in the protein structure upon binding of CsA are reflected in the ^{1}H NMR spectrum of the complex shown in Figure 1A. Comparison of the spectra in Figure 1A,

Table I: Resonance Assignments in the High-Field Region of the ^{1}H NMR Spectrum of Cyclophilin at 25 °C, pH 7.95

resonance	chemical shift (ppm)	resonance assignment
L1	−0.61	Leu_A δ-CH_3
L2	−0.11	Leu_A δ-CH_3
L3	−0.10	CH_3[a]
L4	0.12	Val_A γ-CH_3
L5	0.35	CH_3
L6	0.38	Val_B γ-CH_3
L7	0.41	Leu_A γ-CH
L8	0.50	CH_3
L9	0.56	Val_C γ-CH_3

[a] Resonances labeled CH_3 arise from Val γ-CH_3, Leu δ-CH_3, or Ile γ-CH_3 groups.

1B shows marked rearrangements in the resonance intensities; note in particular the upfield region of the spectrum, which is indicative of CsA binding under conditions of slow exchange on the NMR time scale. Initial estimates from these data of a maximum limit for the rate of the slowest conformational change that occurs on drug binding, $K_{ex} \ll 145$ s^{-1}, appears to be in accord with that expected for the CsA off-rate in the CsA–cyclophilin complex where a dissociation constant of $\approx 3 \times 10^{-8}$ M^{-1} has been determined by Trp fluorescence enhancement measurements (Handschumacher et al., 1984; Harding & Handschumacher, unpublished results). Further analysis of the cyclophilin–CsA spectra will be the subject of a future publication.

Returning now to a more detailed analysis of the ^{1}H NMR spectrum of cyclophilin in the absence of drug, we begin with the most upfield shifted aliphatic resonances where eight distinct resonances were observed in the spectral region located between −0.61 and +0.56 ppm. These resonances were labeled L1–L9 in Figure 2 and Table I since one of the resonances, L2/L3 at −0.11 ppm, is clearly twice the intensity of its neighbors (see Figure 2). The multiplicity of each of these resonances was first established either by direct means, by inspection of heavily resolution-enhanced spectra, or by following their behavior in spin-echo spectra as described in Material and Methods. The results from both approaches were consistent and clearly show that all resonances were doublets with the exception of L7. This feature alone permits assignment of L1–L6, L8, and L9 to methyl resonances. Association of these methyl resonances to specific amino acid spin systems was achieved by the analysis of this region of the spectrum in the 2D COSY and RELAY spectra. The COSY data set (not shown) indicated the following: L1 and L2 were both coupled to L7 which, in turn, was coupled to a resonance at 0.74 ppm; L3 was coupled to 1.45 ppm; L4 was coupled to 1.74 ppm; L5 was coupled to 1.71 ppm; L6 was coupled to 1.82 ppm; L8 was coupled to 1.72 ppm; L9 was coupled to 1.78 ppm. The RELAY data sets are somewhat more revealing because of the observation of additional cross peaks between protons that are not mutually coupled as a result of magnetization transfer through a common coupled proton (Bax & Drobny, 1985). The RELAY data set recorded with a mixing time of 25 ms (Figure 2) showed additional cross peaks between L1 and L2, L4 and 0.96 and 4.19 ppm, L6 and 0.69 and 4.56 ppm, L8 to 1.15 ppm, and L9 and 0.89 and 4.62 ppm. No RELAY peaks were observed for resonance L3 and L5. From these data, L1 and L2 can be assigned to the δ-CH_3 protons of the same Leu residue, since they are both coupled to L7 (Leu γ-CH), and L7 is further coupled to a resonance at 0.74 ppm that must arise from an upfield shifted Leu β-CH proton. L4 and 0.96 ppm are the γ-CH_3 protons of a Val residue, whose β-CH lies at 1.74 ppm and whose α-CH lies at 4.19 ppm. L6 and 0.69 ppm are the γ-CH_3 protons of a

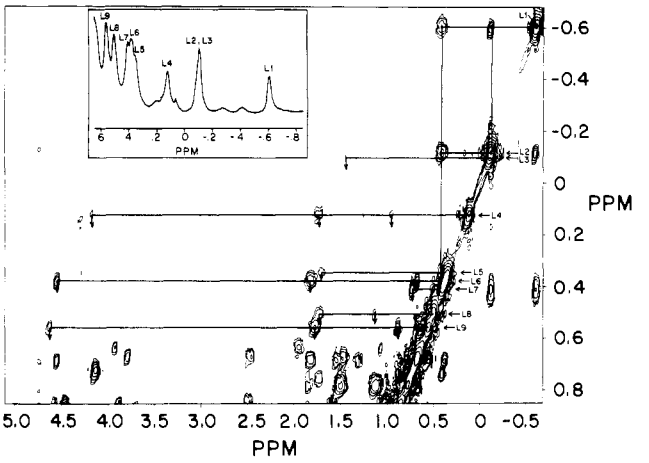

FIGURE 2: Contour plot of 490-MHz ^{1}H RELAY spectrum of cyclophilin (1.7 mM) showing only the upfield methyl RELAY peaks. The insert shows an expanded plot of the upfield methyl region of cyclophilin, with labels indicating the resonances discussed in the text. RELAY parameters: mixing time 25 ms, recycle delay 1.34 s, 378 t_1 points with 144 scans per point, 2K complex free induction decays. Data processed with sine bell window functions to yield a 2K × 2K real matrix (2.94 Hz/point). Solution parameters as in Figure 1.

Val residue with β-CH at 1.82 ppm and α-CH at 4.56 ppm. L8 is either a Val or Leu CH_3 resonance, which shows a RELAY peak to 1.15 ppm (probably a methyl–methyl RELAY cross peak) and is coupled to a resonance at 1.72 ppm. L9 is the γ-CH_3 of a Val residue, with the other γ-CH_3 at 0.89 ppm, the β-CH at 1.78 ppm, and the α-CH at 4.62 ppm. In the absence of information from the RELAY spectrum, L3 and L5 can only be assigned as Val, Leu, or Ile methyl doublet resonances. These assignments and chemical shift values for L1-L9 are summarized in Table I.

We next describe our resonance assignment procedures in the aromatic region of the spectrum (8.2–6.0 ppm). Resonance expected in this region of the cyclophilin spectrum arise from the following residues: 4 His, 15 Phe, 2 Tyr, and 1 Trp. The two singlet resonances (C2H and C4H) arising from each of the four His residues (53, 69, 91, 125) and the singlet arising from the C2H proton of the single Trp residue (120) provide a starting point for these assignments. Of these nine singlet resonances, the His C2H resonances can be expected to resonate to lowest field (Bündi & Wüthrich, 1979). Inspection of a resolution-enhanced spectrum indicated that there were in fact three sharp singlet resonances to the low field of the main aromatic envelope, 8.10, 7.93, and 7.68 ppm, which would seem reasonable, therefore, to assign to three of the four C2H His protons. The locations of the remaining C2H and C4H resonances are not evident in the resolution-enhanced spectrum. In order to locate these singlet resonances, we utilized the spin–echo multiplet selection method described in Materials and Methods to produce a singlet/triplet spectrum. Seven singlet resonances were observed in this spectrum, which include the three resonances assigned above and additional resonances at 7.39, 7.06, 6.87, and 6.68 ppm, thus leaving only two unidentified singlet resonances. In an effort to locate these latter resonances and to associate His C2H and C4H protons with particular ring systems, a pH titration was performed. In these experiments, however, it was necessary to restrict the pH titration to the range of $6.5 < \text{pH} < 8.5$ since irreversible loss of cyclophilin's drug-binding activity occurred outside this range. Considering that His pK_a values in proteins are normally observed to fall between 6 and 7 (Jardetzky & Roberts, 1981), we could at best, therefore, only anticipate observing the initial stages of a pH titration. Indeed, only four

Table II: Resonance Assignments in the Aromatic Region of the ^{1}H NMR Spectrum of Cyclophilin at 25 °C, pH 7.95

spin system	chemical shift (ppm)	resonance assignment
H1	A, 8.10; B, –[c]	His-1[a]
H2	A, 7.93; B, 6.92	His-2
H3	A, 7.68; B, 6.68	His-3
H4	A, 7.39; B, 6.87	His-4
R1	A, 6.15; B, 6.42	Tyr-1
R2	A, 6.19; B, 6.57; C, 6.68	Phe-1
R3	A, 6.24; B, 7.21; C, 7.52	Phe-2
R4	A, 6.66; B, 7.01; C, 7.10	Phe-3
R5	A, 6.68; B, 7.16; C, 7.38; D, 7.71; E, 7.06[b]	Trp-120
R6	A, 6.94; B, 7.23; C, 7.41	Phe-4
R7	A, 7.37; B, 7.58; C, 7.44	Phe-5
R8	A, 6.91; B, 7.29	
R9	A, 6.79; B, 7.02; C, 7.09	Phe-6
R10	A, 7.21; B, 7.34	
R11	A, 7.20; B, 7.31	
R12	A, 6.80; B, 6.93	
R13	A, 6.92; B, 7.05	

[a] For all the His residues, the A resonance is assigned to the C2*H* proton and the B resonance to the C4*H* proton. [b] This resonance is assigned to the C2*H* proton of Trp 120. [c] Dash indicates that the resonance was not found.

singlet aromatic resonances were observed to titrate in this pH range, the C2*H* resonance at 7.93 ppm that titrated in parallel with a previously unidentified singlet resonance at 6.92 ppm and the C2*H* resonance at 7.68 ppm that titrated in parallel with the singlet at 6.68 ppm. These results have been taken as the basis for assigning two of the His spin systems: H2 with C2*H* at 7.93 ppm and C4*H* at 6.92 ppm; H3 with C2*H* at 7.68 ppm and C4*H* at 6.68 ppm. It was possible to carry the correlation of these singlet resonances still further by inspection of the aromatic region of the COSY data set. Despite the small size of the coupling between the C2*H* and C4*H* in His, which makes these resonances appear as singlets in the 1D spectrum, weak scalar coupling cross peaks were observed between the resonances at 7.39 and 6.87 ppm and 7.68 and 6.68 ppm. This confirms the assignment of the H3 spin system deduced from the pH titration and assigns the H4 spin system to C2*H* at 7.39 ppm and C4*H* at 6.87 ppm. The remaining low-field singlet at 8.10 ppm is assigned by default to His H1 C2*H*, leaving the assignment of the remaining singlet at 7.06 ppm to either His H1 C4*H* or Trp120 C2*H* (R5E). Further confirmation of the assignments for the H2, H3, and H4 spin system was obtained from the 250-ms mixing time NOESY spectrum since intraresidue cross-relaxation cross peaks were present for all three of these His residues. Additionally, since no cross peak was observed between 8.10 ppm (H1A) and the singlet at 7.06 ppm in the NOESY spectrum, the 7.06 ppm resonance has been tentatively assigned to the C2*H* proton of Trp 120 (R5E) (vide infra). The location of the H1 C4*H* therefore remains uncertain, although the NOESY data set does show cross peaks between 8.10 and 6.92 ppm and 8.10 and 6.78 ppm. Of these two resonances, 6.92 ppm has previously been assigned to H2 C4*H*; however, at present we cannot exclude the possibility that H1 C4*H* also falls at 6.92 ppm. Since H2 titrates in the range pH 6.5–8.5 and H1 does not, this ambiguity could perhaps be resolved by acquiring a similar NOESY data set at lower pH. These resonance assignments in the aromatic region are tabulated in Table II.

In addition to these singlet aromatic resonances, cyclophilin contains 18 aromatic residues expected to exhibit scalar coupling. These include the 15 Phe residues (6, 7, 21, 24, 35, 45, 52, 59, 66, 82, 87, 111, 112, 128, 144), 2 Tyr residues (47, 78), and 1 Trp residue (120). The problem is now to identify these spin systems in the aromatic region of the spectrum and

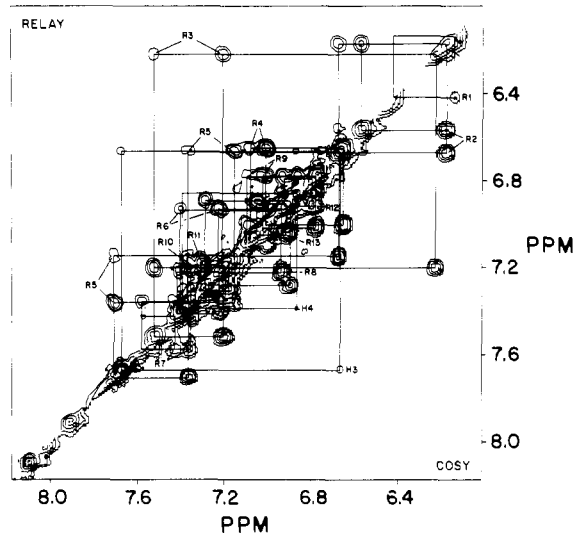

FIGURE 3: Combined contour plot of the aromatic region of the COSY and RELAY spectrum of cyclophilin. RELAY parameters: mixing time 38 ms, recycle delay 1.34 s, 374 t_1 points with 120 scans per point, 2K complex free-induction decays. Data processed with sine bell window functions to yield a 2K × 2K real matrix. COSY parameters: 440 t_1 points with 128 scans per point, 2K complex free induction decays. Data processed with skewed sine bell window functions to yield a 2K × 2K real matrix. Solution parameters as in Figure 1.

assign them initially to amino acid type and then further if possible. Since resonance multiplicities are largely obscured by resonance overlap in the aromatic region of the cyclophilin spectrum, it was necessary to rely on the coupling pattern information derived from the two-dimensional spectra (COSY, RELAY) to distinguish between Phe, Tyr, and Trp spin systems. In the absence of coincidental resonance overlap within these residues and assuming rapid ring flipping of the Tyr and Phe residues, we would anticipate the following patterns: one COSY cross peak and no RELAY cross peak for a Tyr residue; two COSY cross peaks and one RELAY cross peak for a Phe residue; and three COSY cross peaks and two RELAY cross peaks for the Trp residue.

Figure 3 shows a combined plot of the COSY and RELAY spectrum for the aromatic region of cyclophilin. In this case, the RELAY spectrum was recorded with a total mixing time of 38 ms. The coupling patterns derived from the COSY and RELAY spectra are summarized in Table II. Only one entry, R5, in Table II consists of four coupled protons, and therefore, we have assigned this to the Trp-120 residue. At this stage, it is not possible to assign this ring system further since we cannot unambiguously assign R5D (7.71 ppm) or R5A (6.68 ppm) to either the C4*H* or C7*H* (vide infra). Both of these resonances should be doublets, although this could only be confirmed for R5D in a one-dimensional resolution-enhanced spectrum. There are six sets of three coupled protons, R2, R3, R4, R6, R7, and R9, that can be assigned to Phe residues. Within these spin systems, the following protons are coupled to two others in the same Phe ring, allowing for their assignment to the Phe C3, 5*H* protons: R2A, R3B, R4B, R6B, R7B, R9B. Additionally, a resolution-enhanced 1D spectrum reveals that resonances R3C (7.52 ppm) and R2B (6.57 ppm) are doublet resonances, so that the following additional assignments can be made: R2B = C2,6*H*; R2C = C4*H*; R3A = C4*H*; R3C = C2,6*H*. The remainder of the entries in Table II consist of pairs of coupled protons: R1, R8, R10, R11, R12, R13. Two of these pairs are expected to arise from the two Tyr residues while the remainder must arise from Phe residues where coincidental resonance overlap occurs. In the absence of scalar coupling information, however, it is not possible to

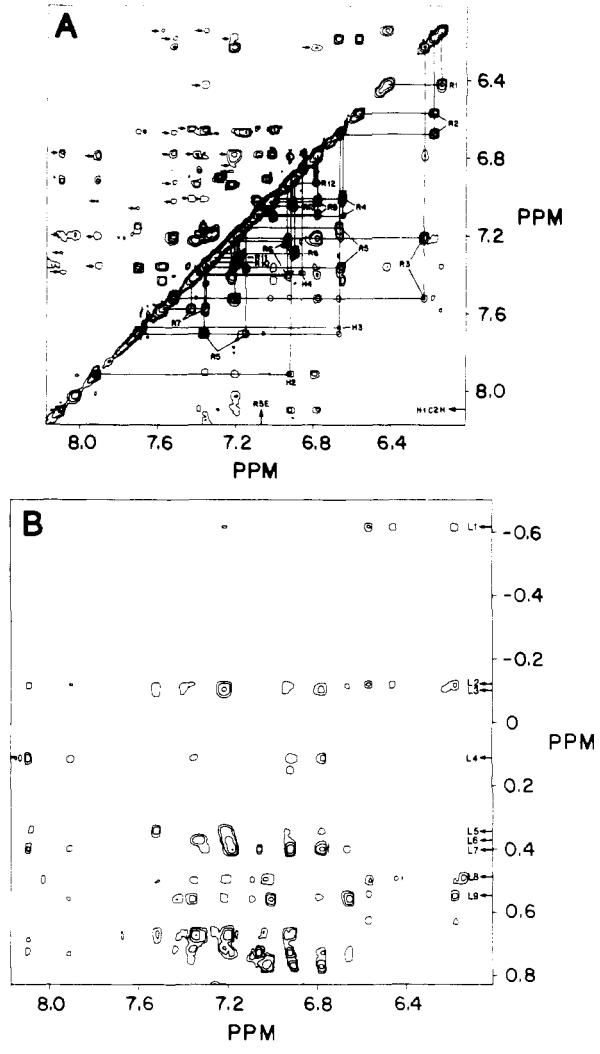

FIGURE 4: Contour plot of pure absorption 490-MHz ^{1}H NOESY spectrum of cyclophilin (1.7 mM) recorded with 250-ms mixing time. Figure 4A shows the aromatic–aromatic region of the data set, with constructs in the lower portion of the figure summarizing the information contained in Table II. In the upper half of the figure, interresidue cross peaks are indicated by an arrow. Figure 4B shows the aromatic to upfield methyl region of the data set, plotted to the same vertical scale as Figure 4A. NOESY parameters: mixing time 250 ± 20 ms, recycle delay 1.69 s, 192 t_1 points with 128 scans real and 128 scans imaginary per point, 2K complex free-induction decays. Data processed with shifted sine bell window functions (20°) to yield a 2K × 2K real matrix (2.94 Hz/point). Solution parameters as in Figure 1.

unequivocally distinguish between these possibilities. However, it is reasonable to tentatively assign the most upfield pair of coupled resonances to a Tyr resonance. Furthermore, if we assume that R1A is the Tyr C3,5*H* protons, then both sets of Tyr protons experience a secondary shift of ~0.7 ppm that presumably results from the proximity of this ring to another aromatic residue (vide infra).

The assignment procedure described above, and summarized in Tables I and II indicates that many aromatic protons and a number of methyl/methylene resonances experience large secondary shifts that presumably arise from ring-current shift effects. In order to determine the clusterings of residues responsible for these shifts, we recorded several NOESY data sets (mixing times 50, 100, and 250 ms). Contour plots for two regions of the NOESY spectrum obtained with a 250-ms mixing time are shown in Figure 4. Figure 4A shows the cross peaks in the aromatic region of the spectrum, and Figure 4B contains the aromatic to upfield methyl cross peaks plotted with the same vertical gain as in Figure 4A. Since the strongest NOESY cross peaks arise from intraresidue cross-relaxation effects, it was possible to use the NOESY spectra, particularly those recorded with the shorter mixing times, to confirm many of the coupling patterns summarized in Tables I and II and hence to distinguish between intra and interresidue cross-relaxation effects. This process is illustrated in Figure 4A where intraresidue cross peaks are indicated in the lower half of the contour plot and interresidue cross peaks are identified by arrows in the upper half of the plot. In Figure 4B, all the observed cross peaks arise from interresidue cross relaxation.

The next stage of the analysis involves the identification of the pairs of protons responsible for each interresidue cross peak. Here we must rely on the ability to assign each proton contributing to a particular cross peak on the basis of the chemical shift of the cross peak in the F_1 and F_2 dimensions. In a protein of this molecular weight, several protons can possess virtually identical chemical shifts, and therefore, an unambiguous assignment of each proton contributing to particular cross peaks may not always be possible. Despite this fact, resonance assignments for several of the interresidue cross peaks can be made, and these data are summarized in Table III for the NOESY data set collected with a 250-ms mixing time. Only one entry in this table is not assignable to an interresidue cross peak, and this is the assignment of the cross peak between 7.06 and 7.71 ppm to R5E (Trp-120 C2*H*) and R5D (Trp-120 C4*H* or C7*H*), respectively. Observation of this cross peak, presumably between Trp C2*H* and C7*H*, confirms the earlier tentative assignment of the singlet at 7.06 ppm (R5E) to Trp-120 C2*H* and, furthermore, allows us to assign the complete Trp-120 spin system (R5) as follows: R5A to Trp-120 C4*H*, R5B to Trp-120 C5*H*, R5C to Trp-120 C6*H*, R5D to Trp-120 C7*H*.

Before the remaining entries in Table III are analyzed in terms of the interresidue distances, some comment about the influence of spin diffusion is warranted in these cyclophilin NOESY data sets (Kumar et al. 1981). In principle, first-order cross-relaxation effects can be distinguished from second-order effects (spin diffusion) by comparison of the mixing time dependence of cross peak intensities. No effort was made, however, to analyze the relative cross peak intensities in these data sets collected with different mixing times. Rather, they were examined only to confirm that essentially all the interresidue cross peaks present in the 100-ms data set were also present in the 250-ms data set. Thus, while it is clear that the influence of spin diffusion should not be disregarded in a quantitative analysis of these NOESY data sets, in the early stages of these studies we have simplified the analysis by assuming that the observation of interresidue cross peaks in the 250-ms NOESY data set reflects an interproton distance of ≤5.0 Å. Another advantage of this qualitative, preliminary approach is that the ambiguities in resonance assigments within an assigned spin system in Table III become unimportant. Furthermore, prior to the sequence-specific assignment of resonances in the cyclophilin ^{1}H NMR spectrum, a more quantitative evaluation of the interresidue distances for input into the distance geometry algorithm of Havel et al. (1983) would be of little value. The interresonance cross-relaxation data contained in Table III were then consolidated into a listing of interresidue distance constraints in Table IV.

Discussion

Assignments of a number of aromatic and high-field methyl resonances in the ^{1}H NMR spectrum of the CsA-free form of cyclophilin have been made on the basis of one-dimensional

Table III: Resonance Assignments for the Interresidue NOESY Cross Peaks in the Aromatic and Upfield-Shifted Methyl Regions of the Spectrum

F_2 resonance	F_1 resonances		
	Aromatic–Aromatic		
6.15, R1A	7.36, R7A(R5C)[a]	7.59, R7B	
6.19, R2A	7.22, R6B	7.52, R3C	
6.24, R3A	6.79, R12A		
6.43, R1B	7.36, R7A(R5C)		
6.66, R4A	7.43, R7C		
6.68, H3C4*H*/R5A[b]	7.30, rc[c]	7.52, R3C	
6.79, R9A	7.22, R3B	7.41, R6C	7.52, R3C
	8.10, H1C2*H*		
6.80, R12A	7.37, R5C(R7A)	7.93, H2C2*H*	
6.84	7.22, R3B		
6.87, H4C4*H*	7.23, R6B		
6.94, R12B	7.52, R3C		
7.01, R4B	7.37, R5C(R7A)	7.43, R7C	
7.02, R9B	7.52, R3C	[7.92, H2C2*H*][d]	
7.06, R5E	7.71, R5D		
7.20, R10A	[8.10, H1C2*H*]		
7.21, R3B	7.92, H2C2*H*		
7.29, R8B/rc	[8.10, H1C2*H*]		
7.37, R7A(R5C)	7.92, H2C2*H*		
7.39, H4C2*H*/rc	8.10, H1C2*H*		
	Methyl–Methyl		
−0.61, L1	0.13, L4	0.51, L8	
−0.11, L2	0.51, L8		
−0.10, L3	0.35, L5		
	Methyl–Aromatic		
−0.61, L1	6.19, R2A	6.46, α*CH*	6.57, R2B
	7.22, R3B		
−0.11, L2	6.19, R2A	6.46, α*CH*	6.57, R2B
	6.67, H3C4*H*/R5A	6.92, H2C4*H*/R12B	6.94, R6A
	7.37, R5C(R7A)	7.40, R6C(H4C4*H*)	7.52, R3C
	7.92, H2C2*H*	8.10, H1C2*H*	
−0.10, L3	6.24, R3A	6.79, R9A	6.94, R6A
	7.22, R3B	7.40, R6C	7.53, R3C
0.12, L4	6.78, R9A	6.92, H2C4*H*/R13A	7.36, R7A(R5C)
	7.92, H2C2*H*	8.10, H1C2*H*	
0.35, L5	6.78, R9A	6.94, R6A	7.21, R3B
	7.52, R3C	8.09, NH	
0.38, L6	7.20, R10A/R11A	7.32, R10B	
0.40, L7	6.67, H3C4*H*/R5A	6.79, R9A	6.92, R12B/H2C4*H*
	7.06, R5E	7.19, R11A	7.31, R11B
	7.92, H2C2*H*	8.10, H1C2*H*	
0.50, L8	6.15, R1A	6.19, R2A	6.45, α*CH*
	6.57, R2B	7.03, (R9B)	7.09, R9C
	7.21, R3B	7.36, R7A(R5C)	7.52, R3C
	8.03 (N*H*)		
0.56, L9	6.19, R2A	6.66, R4A	6.80, R12A
	7.01, R4B	7.10, R4C/R9C	7.22, R6B
	7.36 R7A/R5C	7.42, R7C	7.92, H2C2*H*

[a] Brackets indicate possible alternative resonance assignment. [b] Slash indicates that either resonance assignment is possible. [c] rc denotes random-coil Phe chemical shift. [d] Square bracket indicates a weak NOE.

multiplet selection methods, pH titration experiments, and two-dimensional COSY and RELAY spectra. From these assignments, it was possible to distinguish between inter- and intraresidue cross peaks in selected regions of the NOESY spectrum of cyclophilin and thereby establish the presence of extensive interresidue cross relaxation within and between many of the upfield-shifted methyl and the aromatic resonances. The observation of these interresidue NOE's is indicative of the presence of a cluster of hydrophobic residues within this protein, and inspection of the entries in Table IV reveals that essentially all the spin systems that participate in interresidue cross relaxation also experience large secondary shifts. Specifically, it appears that 11 of the total of 22 aromatic residues, which includes the unique Trp-120 residue, one of the two Tyr residues, two His residues, and seven Phe residues, participate in a hydrophobic cluster that also includes the hydrophobic side chain protons responsible for eight of the nine upfield-shifted aliphatic resonances.

Table IV: Summary of Interresidue Distance Constraints with Interproton Separations of ≤5 Å

aromatic–aromatic	methyl–methyl	methyl–aromatic
R1:R7	L1:L4, L8	L1:R2, R3
R2:R3, R6	L2:L8	L2:R2, R3, R5, R6, H1, H2
R3:R5, R9, R12, H2	L3:L5	
		L3:R3, R6, R9
R4:R5, R7		L4:R7, R9, H1, H2
R5:R12		
		L5:R3, R6, R9
R6:R9, H4		
		L6:R10
R7:H2		
		L7:R5, R9, R11, H1, H2
R9:H1		
R12:H2		L8:R1, R2, R3, R7, R9
		L9:R2, R4, R6, R7, R12, H2

The significance of a compact hydrophobic core in cyclophilin relates to its possible involvement as the site of inter-

action with CsA. Factors that are in favor of this hypothesis are the extent of this cluster, the favorable hydrophobic interaction that could be established with CsA, which itself is a very hydrophobic molecule, and the presence of the Trp residue within this cluster. In regards to the specific environment of the Trp-120 residue within this cluster, examination of Table IV indicates that Trp-120 (R5) is adjacent to three aromatic rings (R3, R4, R12) and two methyl/methine resonances (L2, L7). In Tables I and II, these resonances have been assigned to two and most likely a third Phe ring and a single Leu side chain.

Evidence for a change in the hydrophobic environment of Trp-120 in cyclophilin derived from Trp fluorescence emission studies (Handschumacher et al., 1984). In these latter studies, the enhanced fluorescence emission exhibited by Trp-120 consequent to CsA binding was suggested to result from its exposure to a more hydrophobic environment in the complex. From this data, however, it is not possible to elucidate the structural details of the CsA–cyclophilin interaction or even distinguish between a model where the Trp-120 residue is directly involved in the CsA binding site from one where CsA binds at a site remote from the Trp-120 residues and induces a conformational change that is transmitted to the hydrophobic environment of the Trp residue. These structural details can be resolved by the complete analysis of the two-dimensional ^{1}H NMR data sets on cyclophilin and the CsA–cyclophilin complex. Such studies are currently in progress and include the sequence-specific assignments of spin systems, a more refined analysis of the distance constraints derived from the NOESY data sets, and the use of distance geometry methods for structural modeling.

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