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STRUCTURAL ELEMENTS PERTINENT TO THE INTERACTION OF CYCLOSPORIN A WITH ITS SPECIFIC RECEPTOR PROTEIN, CYCLOPHILIN

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Abstract—Cyclophilin (163 amino acids; 17,737 daltons) is a ubiquitous cytosolic protein that specifically binds the potent immunosuppressive drug cyclosporin A (CsA). To characterize the structural details of this interaction, extensive use has been made of two-dimensional (2D) NMR methods. For studies on CsA, these methods are being used to assign the conformational space accessible to CsA by analysis of the spectra from the multiple CsA conformers present in slow exchange in mixed solvent systems. These same 2D NMR methods also have been used for extensive studies of the major bovine thymus cyclophilin (CyP) isoform and its complex with stoichiometric amounts of CsA. In the former case, these studies have revealed 81% of the 156 expected H_N–H_α crosspeaks. The complete spin-coupled spin systems for one-third of these amide resonances have been assigned according to amino acid type. After exhaustive D_2O exchange, there remain 44 amide protons which exhibit 2D NMR features indicative of a hydrophobic domain with β-sheet secondary structure. The CsA-complexed form of CyP exhibits a discrete structure and set of resonances in slow exchange with the drug-free CyP. The amino acids that have been specifically identified to be affected by the interaction are limited in number and include three Phe residues, the unique Trp at position 120, and two Ala residues.

Cyclosporin A (CsA)** is a hydrophobic, cyclic undecapeptide that exhibits potent immunosuppressive activity [1]. The drug of choice in the prevention of organ and bone marrow transplant rejection, CsA also has shown promising clinical use in the treatment of several autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes mellitus [2]. It has been hypothesized that the molecular mechanism of action for CsA is the inhibition of interleukin-1 and antigen coactivation of T lymphocytes and the blocking of production of interleukin-2, which is required for the differentiation and proliferation of cytolytic T cells [3–6].

A low molecular weight cytosolic protein, cyclophilin (CyP) (17.7 kD), was found to be the specific receptor site for CsA and is responsible for the concentration of CsA by lymphoid and nonlymphoid mammalian cells [7–9]. In addition, subsequent studies have shown that with only two or three exceptions out of more than 100 homologs or metabolites, the binding of cyclosporine analogues to CyP is in direct proportion to their immunosuppressive activity [9]. From these studies, four sequential residues of CsA have been implicated as the particularly important region of the drug that reflects its binding affinity for CyP and conveys the immunosuppressant activity.

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** Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect; DTT, dithiothreitol; DQF, double quantum filter; HOHAHA, homonuclear Hartmann–Hahn; and NOE, nuclear Overhauser effect.

Major and minor isoforms of CyP have been found in all normal and neoplastic mammalian cells tested [10], and CyP has been purified to homogeneity from bovine thymus and human spleen tissues [11]. The major and minor isoforms of bovine and human CyP have been shown to have similar molecular weights, and each specifically binds one molecule of CsA ($K_d \sim 3 \times 10^8\ M^{-1}$) [11]. The complete sequence of bovine CyP (163 amino acid residues) has been determined, and genetic studies have shown that human and rat CyP have greater than 95% sequence identity with bovine CyP [12, 13].

Recently, a very exciting discovery from two other laboratories [14, 15] has shown that bovine cyclophilin is identical in primary sequence to porcine peptidyl-prolyl *cis-trans* isomerase (PPI), and that human and rat CyP have 96% identity to PPI. PPI is responsible for catalyzing the isomerization of the proline imide bond in the peptide backbone of both peptides and proteins and also has been shown to be an abundant cytosolic protein. In view of the seemingly unrelated nature of the proposed function of these two proteins, it is difficult to imagine that the two proteins may be one and the same. However, even more recently, the specific receptor protein of another highly potent immunosuppressive drug, FK506, has been demonstrated to also exhibit significant isomerase activity [16–18]. These results suggest that CyP/PPI may represent the first example of a very interesting protein family whose activity is central to the understanding of the immune system.

Cyclophilin is undoubtedly the primary receptor for CsA, and it is possible that the mechanism of action of CsA in eliciting its immunosuppressive activity is by the inhibition of the enzymatic activity of CyP/PPI. The *cis-trans* isomerization of the proline peptide bond has been implicated at the rate-

determining step in the folding/refolding of proline-containing proteins and, thus, it is interesting to consider the function protein folding may have in the immune response. The isomerase activity of CyP has been shown to be inhibited completely at low concentrations of CsA, and the inhibition constant is in the same range as the dissociation constant of CsA for CyP [15]. Structural models of both the CsA binding site and the enzymatic site of CyP would yield valuable insight into the understanding of a key component in the immune system.

MATERIALS AND METHODS

Protein purification

The major isoform of bovine cyclophilin was isolated from calf thymus and purified to homogeneity as previously described [11] with the following improvements. The major isoform was isolated from the Matrex Blue A affinity chromatography eluate by preparative HPLC chromatography on a Waters ACCELL carboxymethyl cation exchange resin with isocratic elution (5 mM potassium phosphate, 45 mM NaCl, pH 6.9, at 6 mL/min). The protein was then stored at 4°, 200 mM NaCl, 5 mM β-mercaptoethanol, pH 7.2. Ultrapure cyclophilin samples were obtained by further purification on an analytical TSK G/2000 size exclusion HPLC column (20 mM potassium phosphate, 200 mM NaCl, pH 6.8). NMR samples were prepared by concentrating the sample (~20–40 mg) in an Amicon MC-10 ultrafiltration unit fitted with a YM-10 membrane to 5 mL, dialyzing the sample into the desired buffer (20 mM potassium phosphate, 200–300 mM NaCl, 2 mM dithiothreitol, pH 6.8), and then concentrating the sample by ultrafiltration under N_2 to a final volume of 400 μL. Samples in the NMR tubes were flushed with argon and sealed with a cap.

The CyP : CsA complex was generated by agitating a purified protein sample in the presence of two molar equivalents of pulverized CsA (Sandoz, used without further purification) for a period of 4–6 hr at 6°. Excess CsA was removed by centrifugation, and the supernatant fraction was transferred to an NMR tube and treated as above.

The NMR-labeled CsA derivatives were synthesized at Merck, Sharp & Dohme Laboratories, by P. Durette and used without further purification.

NMR methods

One- and two-dimensional NMR spectra were collected on a Bruker AM500 NMR spectrometer. All datasets were processed using the FTNMR software package from Hare Research, Inc., on a Sun 4/280S. Correlated spectroscopy (COSY [19]) experiments were run in the absolute-value mode, while double quantum filtered COSY (DQF-COSY [20]), phase-sensitive COSY [21], homonuclear Hartmann–Hahn (HOHAHA [22–24]), and nuclear Overhauser effect (NOESY [25]) experiments were run in absorption mode utilizing time-proportional phase incrementation (TPPI [21]). Phase-cycling permitted quadrature detection in t_1, therefore, the spectrometer carrier offset was placed on the water solvent resonance. Two-dimensional spectra were collected as 2048 complex points in t_2 with 400–530 t_1 values. Water suppression was achieved by solvent presaturation, and in some experiments a composite 90° read pulse [26] was used for baseline improvement. Composite 90° pulses were used for the double quantum filter in the phase-sensitive COSY sequence [27], and the mixing time in the NOESY experiments was randomly varied by 10% to reduce coherence transfer effects [28]. Final two-dimensional matrices were left unsymmetrized.

RESULTS AND DISCUSSION

Studies of cyclosporin A

The sequence of the cyclic undecapeptide, CsA, is comprised of several unusual amino acids which include: (4*R*) - *N* - methyl - 4 - butenyl - L - threonine (MeBmt) at position 1, α-aminobutyric acid (Abu) at position 2, sarcosine (MeGly) at position 3, *N*-methylleucine (MeLeu) at positions 4, 9 and 10, and *N*-methylvaline (MeVal) at position 11. As would be expected from the hydrophobic nature of these amino acids, CsA is only sparingly soluble in water. However, due to the relatively small size of this cyclic peptide and its solubility in aprotic solvents, CsA has been a popular molecule for NMR studies and its structure has been determined in organic solvents [29]. For the most part, this structure shares many similarities with the previously solved crystal structure [30]. The structure of CsA, determined by X-ray crystallography, is shown in Fig. 1 with the four residues vital to CyP binding highlighted.

The conformation of CsA when complexed to CyP most probably will be different from the structure determined in organic solvents, even though there is evidence that CsA binds in a hydrophobic region of CyP [31]. A strategy that we are using in our structural studies of CsA is to experimentally probe the conformational space accessible to CsA through the use of mixed solvents where multiple CsA conformations are present in slow exchange. An example of this is illustrated for a 50/50 mixture of methanol/water where eight different conformations were found in the full analysis of the DQF-COSY spectrum shown in Fig. 2. For structural features, NOESY spectra were also collected, and it was interesting to note that for one of the fully assigned conformers, the NOE between the alpha protons of MeLeu9 and MeLeu10 was lost. This NOE was the signature for the *cis* peptide bond that was found in both the crystal structure and the NMR structure obtained in organic solvents. It is also worth mentioning that, in this mixed solvent system, the most significant changes with respect to previous NMR studies in neat solvents appeared clustered in the region that has been implicated previously as the "active surface," namely residues MeBmt1, Abu2, MeLeu10 and MeVal11.

Studies of cyclophilin

In addition to bovine cyclophilin, the complete primary sequences of human and rat cyclophilins have been determined [12, 13]. The sequence homologies are shown in Fig. 3. The two isoforms of

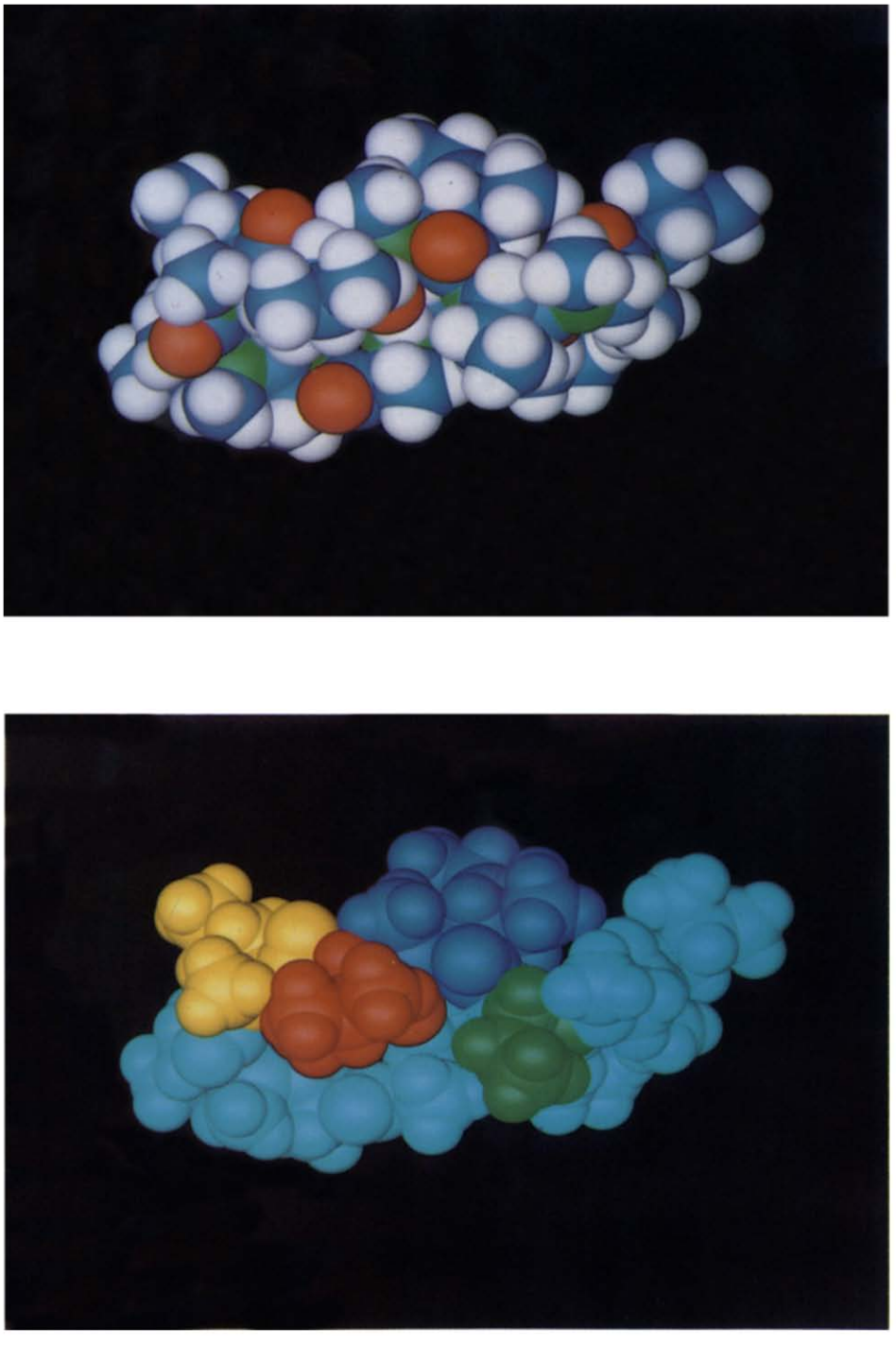

Fig. 1. Upper panel: Spacefilling model of CsA based on the X-ray crystal structure. Carbon atoms have been colored blue, nitrogen atoms green, oxygen atoms red, and protons white. Lower panel: The "active surface" of CsA has been highlighted (same orientation as above). The MeBmt1 residue is blue, Abu2 is green, MeLeu10 is yellow and MeVal11 is red. These figures and Fig. 9 were generated using the Sybyl software package (Tripos Associates) on a Sun 4/280S and displayed on an Evans and Sutherland PS390.

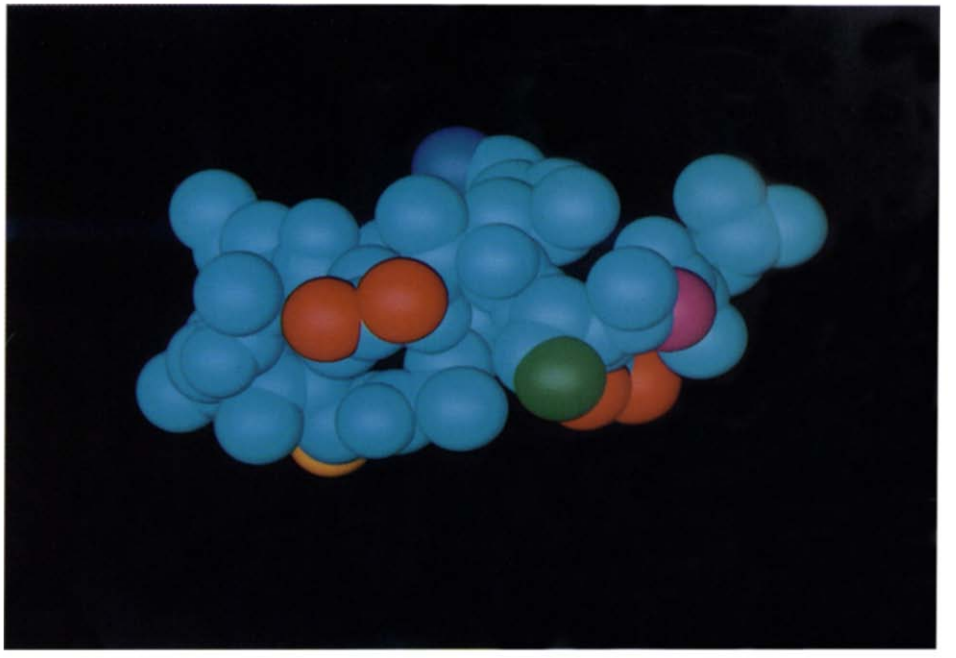

Fig. 9. Spacefilling model of CsA to show the NMR-labeled CsA derivatives to be used in our studies (same orientation as in Fig. 1 except that the protons have been deleted). The η carbon of MeBmt1 (blue) has been labeled with ^{13}C. The γ methyl group of Abu2 (green) has been both mono- and di-fluoro substituted. The side chain of Sar3 (magenta) has been extended by a methyl group which is ^{13}C labeled. All four γ methyl groups of Val5 and MeVal11 (red) have been individually mono-fluoro substituted. The Ala8 residue (yellow) has been substituted with a lysine with a nitroxide spin label attached at N_ξ.

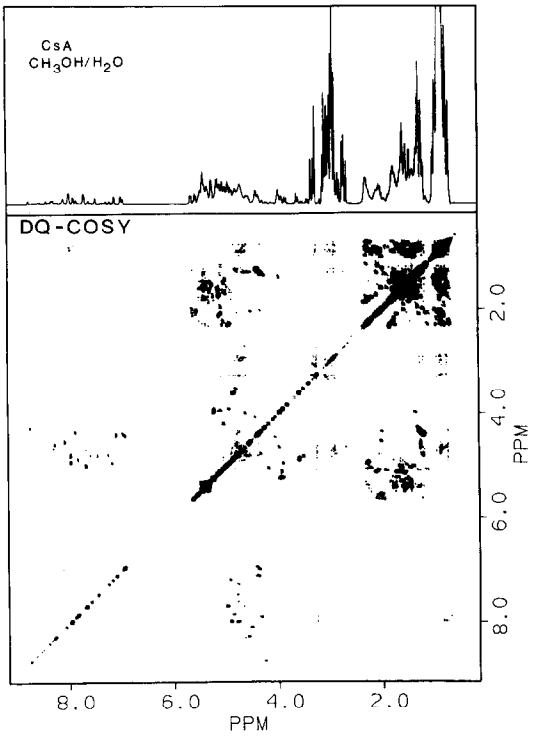

Fig. 2. DQF-COSY spectrum of a 16 mM CsA sample in CD_3OD/D_2O (1:1). The one-dimensional spectrum is shown along the top. A dataset size of 512 × 2048 was collected.

bovine cyclophilin, which both bind one molecule of CsA, have the same primary sequence and differ only in that the minor form is acetylated at the N-terminus. Physical chemical studies have suggested that the CsA-binding activity of cyclophilin is sulfhydryl dependent and that a single Cys residue may be an important contributor to CsA binding [15].

Published NMR studies from this laboratory have been directed towards the preliminary structural characterizations of cyclophilin and the cyclophilin: cyclosporine complex [32, 33]. Our long-term objective is to elucidate the complete solution structure of this receptor–drug complex. Ultimately, this will necessitate the complete sequential NMR assignment of the amino acid residues in this protein. However, the large size (on the NMR scale!) of this protein may prevent our determining a complete sequential resonance assignment without the use of selective and/or complete isotopic enrichment methods [34–41]. It is of note, therefore, to acknowledge that the cyclophilin gene has been isolated, and collaborative work has been initiated to find an optimal expression system for the protein in order to produce the NMR quantities of isotopically-labeled protein samples needed for these studies. With this activity in progress, we are continuing to use the native bovine CyP to identify regions of structure in the protein and, more specifically, to determine the identity of the amino acid residues of the protein that are involved in the binding of cyclosporine. The discussion that follows will highlight our NMR studies to date.

```
                                        10                                  20
Bovine      Val-Asn-Pro-Thr-Val-Phe-Phe-Asp-Ile-Ala-Val-Asp-Gly-Glu-Pro-Leu-Gly-Arg-Val-Ser
r-HUMAN Met-  "                          "                                   "
RAT                                     Thr-Ala                             Cys

                                        30                                  40
            Phe-Glu-Leu-Phe-Ala-Asp-Lys-Val-Pro-Lys-Thr-Ala-Glu-Asn-Phe-Arg-Ala-Leu-Ser-Thr

                                        50                                  60
            Gly-Glu-Lys-Gly-Phe-Gly-Tyr-Lys-Gly-Ser-Cys-Phe-His-Arg-Ile-Ile-Pro-Gly-Phe-Met
                                                 "
                                              Ser
                                    70                                      80
             Cys-Gln-Gly-Gly-Asp-Phe-Thr-Arg-His-Asn-Gly-Thr-Gly-Gly-Lys-Ser-Ile-Tyr-Gly-Glu

                                        90                                  100
            Lys-Phe-Asp-Asp-Glu-Asn-Phe-Ile-Leu-Lys-His-Thr-Gly-Pro-Gly-Ile-Leu-Ser-Met-Ala
                   Glu
                   Glu
                                   110                                      120
          Asn-Ala-Gly-Pro-Asn-Thr-Asn-Gly-Ser-Gln-Phe-Phe-Ile-Cys-Thr-Ala-Lys-Thr-Glu-Trp

                                       130                                  140
            Leu-Asp-Gly-Lys-His-Val-Val-Phe-Gly-Lys-Val-Lys-Glu-Gly-Met-Asn-Ile-Val-Glu-Ala
                                                                 "
                                                              Ser
                                       150                                  160
            Met-Glu-Arg-Phe-Gly-Ser-Arg-Asn-Gly-Lys-Thr-Ser-Lys-Lys-Ile-Thr-Ile-Ala-Asp-Cys
                                                                           "
                                                                        Ser
                  163
            Gly-Gln-Ile
                  -Leu-Glu
                   Ile
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Fig. 3. Comparison of the primary sequences of three cyclophilins. The major bovine isoform is regarded as the reference, and differences are indicated where appropriate. The amino acids are identified by their three letter abbreviations.

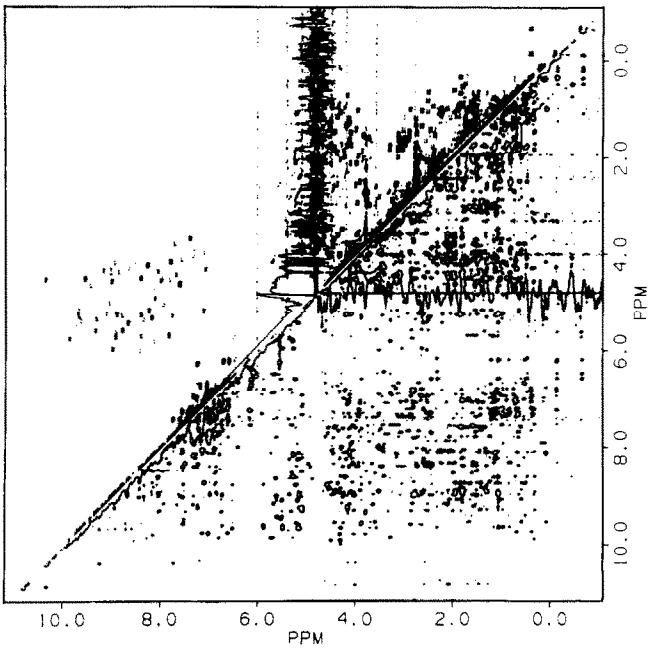

Fig. 4. A composite two-dimensional ^{1}H NMR spectrum of bovine CyP at 25°. The DQF-COSY spectrum is shown above the diagonal and the NOESY occupies the area below the diagonal. The sample used for the DQF-COSY experiment was 3.0 mM protein in 90% H_2O/10% D_2O, 10 mM potassium phosphate (KP_i), 300 mM NaCl, 10 mM DTT, pH 6.8. A total of 128 scans were taken per t_1 value, and a dataset size of 512 × 2048 was collected. The sample used for the NOESY experiment was 2.3 mM protein, 10 mM KP_i, 200 mM NaCl, 2 mM DTT, pH 6.8. A total of 160 scans were taken per t_1 value, and a dataset size of 512 × 2048 was collected. The mixing time, nominally 175 msec, was randomly varied by 10% to reduce coherence transfer effects.

A composite of the two-dimensional ^{1}H NMR spectra obtained for the drug-free protein is shown in Fig. 4, in which the spectrum above the diagonal is from a double quantum filtered COSY experiment and the spectrum below the diagonal is from a NOESY experiment. The dispersion of chemical shifts in the aliphatic region of the DQF-COSY experiment facilitated the identification of many amino acid spin systems and, as can be seen in the NOESY spectrum, there is a wealth of structural information to be extracted. Essential to making sequential assignments is a well resolved amide-H_α fingerprint region. In our two-dimensional spectra obtained in H_2O, we have observed 126 of the 156 expected crosspeaks in the fingerprint region (Gly pairs counted as one). Thus far, 43 of the 126 observed amide-H_α crosspeaks have been classified according to amino acid type. After exhaustive exchange in D_2O, there are still 44 crosspeaks remaining in the fingerprint region. Of the 126 observed amide peaks, 33 have been assigned to hydrophobic residues and of these, 15 are retained in the D_2O spectra. These persistent amides suggest the presence of compact, well-defined structures in this protein which, on the basis of our preliminary analysis, appears to be a hydrophobic domain with β-sheet secondary structure [33].

Our preliminary studies gave evidence for the existence of a hydrophobic core within the protein and, on the basis of fluorescence experiments in which the emission of the tryptophan fluorophore was monitored as a function of CsA binding, this hydrophobic region was implicated in the interaction with CsA [31]. As a result, our efforts have been concentrated towards the hydrophobic residues which constitute 50% of the protein. Of these, the most progress has been made with the aromatic residues. All of the aromatic ring spin systems for the 15 Phe, 4 His, 2 Tyr and 1 Trp have been assigned with the exception of one Phe and His. As can be seen in the NOESY spectrum of the free protein (Fig. 5), there are many nonsequential NOE interactions between these hydrophobic amino acids moieties and other aliphatic protons.

Studies of the cyclophilin:cyclosporin A complex

Our NMR studies on the CyP:CsA complex have demonstrated that there are some significant selective changes in chemical shifts. It has been shown that cyclosporine binds stoichiometrically very tightly to cyclophilin ($K_d \sim 3 \times 10^8\ M^{-1}$) and is in the regime of slow exchange on the NMR timescale (Fig. 6). In our two-dimensional spectra of the complex, 104 of the 160 expected amide-H_α crosspeaks (156 from CyP, Gly pairs counted as one; 4 from CsA) are observed. Of these crosspeaks, 24 are unique in comparison to the spectra of the drug-free protein. In comparisons of the aromatic regions of the spectra, four of the assigned aromatic spin systems are clearly shifted and the largest effect occurs for the Trp ring NH proton. In the NOESY spectrum of the drug-free protein, there were only two NOEs

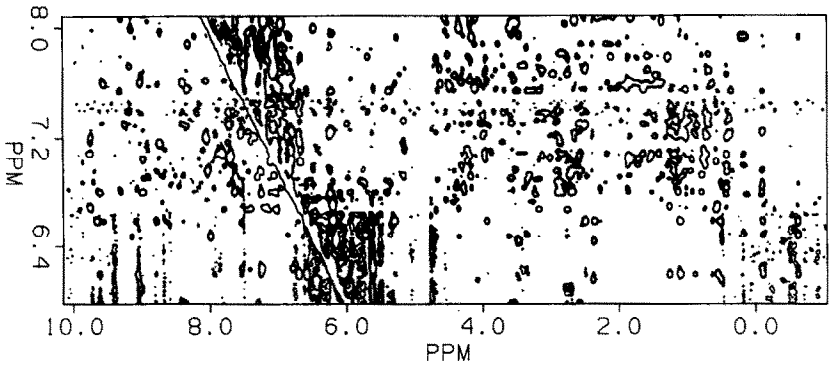

Fig. 5. Expansion of the NOESY spectrum in Fig. 4. This portion of the spectrum shows the NOE interactions between the aliphatic protons and the aromatic residue protons.

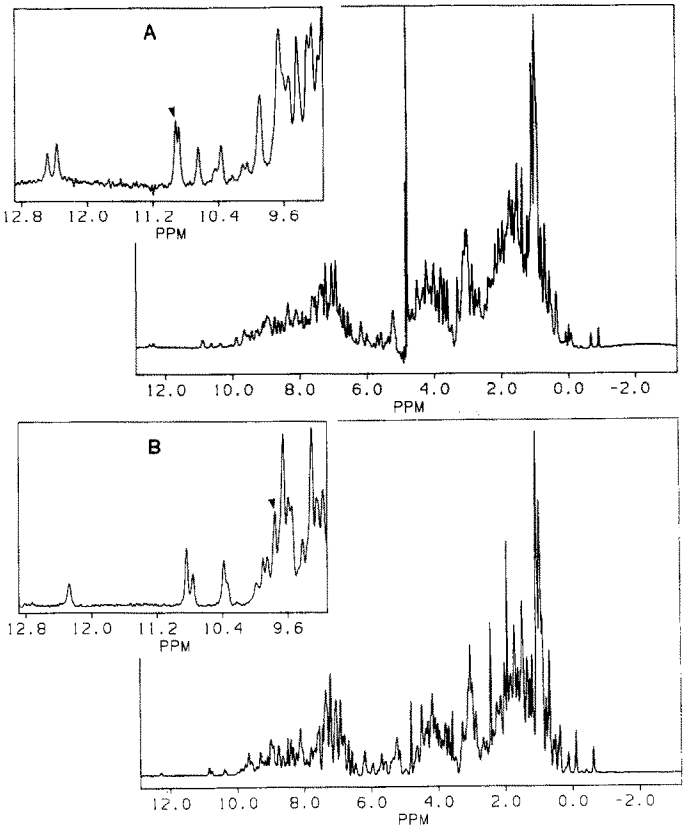

Fig. 6. One-dimensional spectra of CsA-complexed CyP (A) and drug-free CyP (B). The CyP:CsA complex was generated as described in the text. The CyP concentration in the complex was 1.7 mM in 90% H_2O/10% D_2O, 10 mM KP_i, 200 mM NaCl, 2 mM DTT, pH 6.8. The sample used to obtain the spectrum (B) was the same sample that was used to obtain the NOESY spectrum in Fig. 4. The insets show the downfield aromatic protons. The Trp120 ring NH proton, identified with an arrow, has shifted downfield by 1.15 ppm in the complex.

from the Trp NH proton to the upfield methyl region of the spectrum (Fig. 7A). In the CyP:CsA complex (Fig. 7B), this proton shows 6 NOEs to the methyl region of the spectrum! Possible interactions and conformational changes in the local Trp environment consequent to CsA binding as seen in these NMR experiments are corroborated and consistent with previous steady-state fluorescence experiments that showed a 2-fold increase in Trp fluorescence emission upon drug binding.

Presumably, some of the numerous NOEs observed between aromatic spin systems and methyl resonances in the complex reflect intermolecular interactions between the protein residues and the residues of the bound drug. A complete resonance assignment of the free drug in aqueous solution

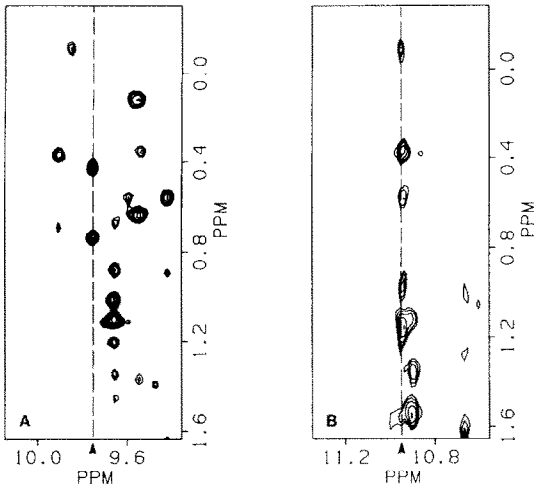

Fig. 7. Expansion of the NOESY spectra from the drug-free CyP (A) and the CsA-complexed CyP (B) samples as described in the legend of Fig. 6. The region plotted shows the NOE interactions between the Trp120 ring NH proton (indicated by the broken line) and the upfield methyl region.

would definitely facilitate resonance assignments arising from the bound drug; however, due to very limited solubility of CsA in H_2O, all reported NMR studies with CsA have been performed in non-aqueous solvents. Recently, we have been successful in obtaining some reasonable spectra of CsA in H_2O as shown in the one-dimensional spectrum in Fig. 8. Interestingly, contrary to what might be expected from the conformational studies in mixed solvents, only one major conformation of the drug was observed. Analysis of the two-dimensional spectra is currently underway.

We have also initiated a concerted set of studies directed towards extending the identification of residues that are present in the binding region of cyclophilin through the use of NMR-labeled CsA analogues and the use of one- and two-dimensional heteronuclear NOE experiments [42–46]. The analogues of CsA that we are using include CsA derivatives that are specifically labeled with ^{13}C and ^{19}F, as well as an attached nitroxide spin label. The derivatives available are shown in Fig. 9. The use of these analogues and the isotope directed NOE techniques focus only on the binding domain and are being conducted prior to having a complete sequential assignment!

Studies of cyclophilin isomerase activity

Of great interest is the enzymatic isomerase activity that is common to both cyclophilin and the receptor protein of the immunosuppressant drug FK506 [17, 18]. These are the first examples of perhaps a new family of proteins that not only specifically bind immunosuppressive drugs, but also exhibit peptidyl prolyl isomerase activity, suggesting that there is much to be learned about the role of protein folding in the response of the immune system. The first question that we hope to answer in regards to the enzymatic activity of CyP concerns the location of its active site. Does the active site for prolyl isomerization occur in the same region as immunosuppressive drug binding? This will be studied by both fluorescence spectroscopy and NMR experiments. The NMR experiments will involve the use of both unlabeled and NMR-labeled proline-containing peptide substances, analogous to what is currently in progress with the CsA drug derivatives. We will also be investigating the possibility of similar conformation changes that may occur upon CsA drug

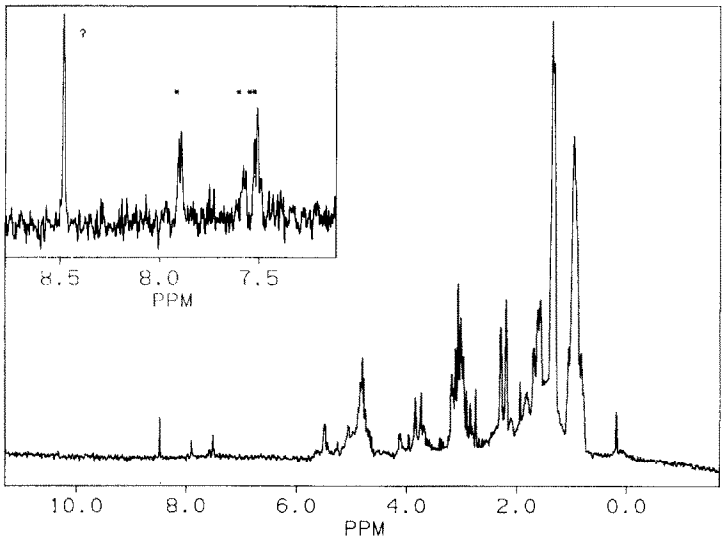

Fig. 8. One-dimensional spectrum of CsA in D_2O, pH 7.5. A total of 2048 transients were collected with solvent presaturation. An expansion of the amide region is shown in the inset. Amide resonances are indicated with an asterisk, and an unidentified peak is indicated with a question mark.

binding and prolyl isomerase activity. From these studies, we hope to determine whether the inhibition of isomerase activity by CsA binding is due to competitive inhibition or to a change in the structural conformation of cyclophilin which precludes accessibility to the enzymatic site.

Overview

While still in its infancy, our NMR studies on cyclosporine, cyclophilin and the CyP:CsA complex have revealed some structural information that will be useful in the full elucidation of the structural details of the CyP:CsA interaction. The primary limiting factor to a complete structural elucidation of the free and drug-bound protein by NMR methods is the large size of the protein, as would be expected. We have, however, been able to overcome this problem to a certain extent by improving the isolation, purification and stability of the protein, as well as the optimization of the experimental data collection methods and processing. Although the line broadening concomitant with increasing molecular weight has made the identification of some spin systems difficult, the combined use of COSY, HOHAHA and NOESY spectra has enabled the assignment of over 30% of the observed amide resonances according to amino acid type. Similar assignment strategies were applied to the spectra of the CyP:CsA complex with similar results.

Comparison of the spectra from the free and CsA-complexed protein not only assisted in the assignment of spin systems, but also revealed that relatively few proton resonances were appreciably affected by complexation. Most of the residues affected by drug binding have been identified as being a structural hydrophobic domain of the protein with slow amide exchange rates. These results suggest that CsA, an extremely hydrophobic molecule, may bind to a region of the protein that is not readily accessible to water. In fact, the patterns of NOE interactions from this domain in the two-dimensional spectra are consistent with a β-sheet conformation and may involve a mixture of parallel and anti-parallel β-strands.

The conformation of CsA in organic solvents has been described as a twisted β-sheet [29]; therefore, the interaction of CyP with CsA may occur by sheet-to-sheet interactions. To test this structural hypothesis, we have been investigating the conformational space available to CsA by the use of mixed solvents, mimicking an environment of differing lipophilicity. However, to ultimately resolve the CsA binding site in CyP requires the assignment of those resonances arising from CsA in the spectra of the CyP:CsA complex. To assist in this effort, we are currently attempting to establish the conformation of CsA free in aqueous solution.

At this time there is not enough information to definitively assign the CsA-binding region of CyP or to comment on whether the binding site is the same as the site for the biological activity of CyP. The use of NMR-labeled CsA analogues should assist in resolving specific residues in the protein-bound CsA complex, and the use of isotope-directed NOE studies should establish which residues interact with CyP. Similar strategies will also be adopted with peptidic isomerase substrates with the goal of resolving some of the questions that have arisen recently with respect to the isomerase activity of CyP. Identifying the CsA binding site and the enzymatic site of CyP is a necessary first step in understanding the role that this protein plays in the immunological response.

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