Solution Structure of Cyclosporin A and a Nonimmunosuppressive Analog Bound to Fully Deuterated Cyclophilin†

Victor L. Hsu‡ and Ian M. Armitage*,§

Departments of Molecular Biophysics and Biochemistry, Pharmacology, and Diagnostic Radiology, Yale University, 333 Cedar Street, P.O. Box 3333, New Haven, Connecticut 06510

Received July 9, 1992; Revised Manuscript Received October 14, 1992

ABSTRACT: A simple strategy involving ^1H nuclear magnetic resonance (NMR) spectroscopy and complete protein deuteration was used to determine the structures of two receptor-bound drugs. A potent immunosuppressive, cyclosporin A (CsA) binds tightly to the ubiquitous and highly conserved 17.7-kDa immunophilin, cyclophilin (CyP). Fully deuterated CyP was produced by overexpressing the human CyP gene in *Escherichia coli* grown on deuterated algal hydrolysate in 98% D_2O. As only the CsA molecule is protonated in the CsA–CyP complex, we were able to make a complete sequential assignment of the bound drug using standard two-dimensional proton NMR experiments. The structure determination was accomplished using dynamical simulated annealing calculations with a total of 124 NMR-derived distance and torsion angle restraints. Aside from binding CsA, CyP also acts as a peptidyl-prolyl cis–trans isomerase. Thus, much importance had been ascribed to the cis peptide bond present in the structures reported for free CsA in organic solvents and in crystal studies. Interestingly, CyP-bound CsA exists in an all-trans conformation with no detectable elements of regular secondary structure and no intramolecular hydrogen bonds. A nonactive CsA analog, MeAla6-CsA, was studied using the same CyP deuteration strategy. In addition to structural elucidation of the two bound drugs, we were able to differentiate between the bound and surface-exposed residues of the drugs and also validate our previous hypothesis that the single CyP tryptophan is located in the CsA-binding site. The total backbone rms deviation of the average structures of the two bound drugs was 0.54 Å with the primary structural difference arising from the single amino acid substitution which occurs on the solvent-exposed surface of the bound drugs. This supports recent studies which postulate that immunosuppressive drug activity may be mediated by this "effector surface". This method of complete protein deuteration greatly facilitates the conformational elucidation of receptor-bound drugs and identifying specific sites of intermolecular interactions and should also find great utility in detailed structural studies of other receptor–ligand complexes.

Cyclosporin A (CsA),[1] a hydrophobic cyclic undecapeptide which exhibits potent immunosuppressive activity, is currently the drug of choice in the prevention of organ and bone marrow transplant rejection. It has been hypothesized that the mechanism of action of CsA involves 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 cytosolic T cells (Elliot et al., 1984; Kronke et al., 1984). A low molecular weight cytosolic protein, cyclophilin (CyP), was found to be a high-affinity binding protein for CsA and is responsible for the concentration of CsA by lymphoid and nonlymphoid mammalian cells (Handschumacher et al., 1984; Koletsky et al., 1986). A highly conserved, ubiquitous protein that has been isolated in large abundance in species ranging from *Escherichia coli* to tomato plants to humans, CyP also possesses proline cis–trans isomerase activity which is inhibited by CsA (Fischer et al., 1989; Takahashi et al., 1989). Consequently, interruptions in proline isomerism and/or protein folding were implicated as potential modes of immunosuppression by CsA. This possibility of a new mechanism of signal transduction was strengthened when it was discovered that a specific binding protein for another potent immunosuppressant, FK506, was also a proline isomerase (Harding et al., 1989; Siekierka et al., 1989). A recent finding that the chemically and structurally unrelated drugs CsA and FK506 both facilitate the interaction of their specific immunophilins (CyP and FKBP, respectively) with a common enzyme target (Liu et al., 1991) suggests that the structure of the solvent-exposed surface of the immunophilin-bound drugs may play a pivotal role in controlling immunosuppressive activity by mediating multicomponent interactions.

Herein we report upon the receptor-bound solution structures for both CsA and a nonimmunosuppresive CsA analog as determined by standard two-dimensional (2D) homonuclear NMR techniques and simulated dynamic annealing using fully deuterated CyP. As a technique that permits accurate solution structure determination of biomolecules at angstrom resolution, NMR spectroscopy should prove invaluable to pursuing the rational design of drugs. Unfortunately, a complete sequential assignment of the free CyP protein has not been reported, and due to the hydrophobic nature of CsA, the sequential assignments reported for this drug free in solution have thus far been attained only in organic solvents (Kessler et al., 1985). Several elegant NMR experiments have been

† This work was supported in part by grants from the NIH (GM 40660 and DK 18778) and a James Hudson Brown–Alexander B. Coxe Fellowship (to V.L.H.) NMR instrumentation and computational facilities were provided by grants from the NIH (RR 03475), NSF (DMB 8610557), and the American Cancer Society (RD 259).

‡ Department of Molecular Biophysics and Biochemistry. Present address: Department of Chemistry, B–042, University of California, San Diego, La Jolla, CA 92093-0342.

§ Departments of Pharmacology and Diagnostic Radiology.

[1] Abbreviations: 2D, two-dimensional; 3D, three-dimensional; 4D, four-dimensional; COSY, correlated spectroscopy; CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506 binding protein; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

0006-2960/92/0431-12778$03.00/0

developed which are based on the detection of only those protons that are attached to NMR-active isotopes of other nuclei such as ^{13}C and ^{15}N. Labeling proteins with these nuclei has made it possible to study larger proteins and protein complexes by adding extra dimensions to NMR (3D and 4D). These two- and three-dimensional heteronuclear NMR methods have recently been used to determine the structure of CyP–bound CsA (Fesik et al., 1991; Weber et al., 1991). Since the number of proton resonances arising from CsA comprises only a small percentage of the total number of resonances in the CsA–CyP complex, we chose to adopt a different nuclei-filtering strategy to determine the structure of CsA and a unique analog bound to CyP. This was achieved by the production of fully deuterated recombinant human CyP (163 amino acids, 17.7 kDa) in which all of the nonexchangeable protons are deuterated. Fully deuterated CyP has no protons, and therefore the only resonances that are observed are those from the bound drug.

An ideal way to understand the relationship between the structure and activity of a drug is to compare structural features of a known active drug with a nonactive analog. We selected one such analog of CsA in which the *N*-methylleucine (MeLeu) residue at position 6 was substituted with *N*-methylalanine (MeAla). This particular drug analog (MeAla6-CsA) has been shown to bind tightly to CyP but does not exhibit immunosuppressive activity (Durette et al., 1988).

MATERIALS AND METHODS

Materials. The recombinant human cyclophilin gene, cloned into the pHN1+ expression vector, was kindly provided by S. Schreiber and C. Walsh (Liu et al., 1990) and was subsequentially transformed into the *E. coli* host XA90 F′ *lacI*^{Q1}. Fully deuterated CyP was produced by growing the bacteria and expressing the protein in deuterated algal hydrolysate in D_2O, generously provided by the Martek Corp. (Columbia, MD). We found it advantageous to first adapt the host bacteria for growth in a deuterated environment before transformation. This was accomplished in three steps of 70, 90, and 98% D_2O. At each of these levels XA90 *E. coli* was grown in Luria–Bertani (LB) medium made with the appropriate level of D_2O enrichment. The bacteria were incubated with shaking at 37 °C until A_{600} reached 0.7, at which time an aliquot was transferred to an appropriately deuterated agar plate and incubated at 37 °C. Single colonies of "D_2O capable" bacteria were then isolated and grown at the next higher level of D_2O enrichment. Due to deuterium isotope effects, bacterial growth in LB media and on the agar plates was slowed by approximately a factor of 2. After adaptation to 98% D_2O, the bacteria were made competent and transformed using standard calcium chloride methods (Maniatis et al., 1982).

A 1-L culture of XA90/pHNJ was grown at 37 °C with shaking in deuterated algal hydrolysate in D_2O (98% D) (Celtone-D, Martek Corp.) containing 100 μg/mL ampicillin. Protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (from a sterile D_2O stock solution) to a final concentration of 1.5 mM when A_{600} reached 0.8 (after approximately 7 h). Incubation was allowed to continue for an additional 14–16 h. Although growth and expression was again slowed by a factor of 2, longer induction times did not increase yield and an overabundance of β-lactamase became a concern. Purification of the deuterated protein was the same as for normal protonated protein (Liu et al., 1990) and was fully active as quantitated by our LH-20 column assays (Handschumacher et al., 1984). Cyclosporin A samples were from R. E. Handschumacher, and the MeAla6-CsA derivative was from P. Durette at Merck, Sharp & Dohme Laboratories.

NMR Spectroscopy. Samples were prepared as previously described (Heald et al., 1990) and ultrafiltered into the final buffer of 20 mM phosphate and 250 mM NaCl, pH 6.8. The complexes were formed in the presence of excess protein to ensure that all CsA molecules were bound.

All spectra were collected on a Bruker AM500 NMR spectrometer at 15–30 °C. Data sets were processed and analyzed using the FTNMR software package from Hare Research, Inc., on a Sun 4/280S, SparcStation 1+, or a Silicon Graphics 4D/25TG. The identification of amino acid spin systems and the complete sequential assignments for both drugs were accomplished with the combined use of two-dimensional phase-sensitive correlation spectroscopy (COSY) (Marion & Wüthrich, 1983), double-quantum-filtered COSY (Rance et al., 1983), homonuclear Hartmann–Hahn spectroscopy (HOHAHA, also called TOCSY) (Bax & Davis, 1985), and nuclear Overhauser effect spectroscopy (NOESY) (Macura & Ernst, 1980). Phase cycling enabled quadrature detection in t_1 and employed time-proportional phase incrementation (TPPI) in the absorption mode (Marion & Wüthrich, 1983). The spectra consisted of 2048 complex data points in t_2 and were acquired with the carrier frequency placed at the HOD resonance. Water suppression was achieved by solvent presaturation. A composite 90° read pulse was used in some experiments to minimize baseline distortion (Bax, 1985), and the mixing time in the NOESY experiments was randomly varied by 10% to reduce coherence transfer effects (Macura et al., 1982).

Structure Calculations. The structure determination was accomplished using a dynamic simulated annealing protocol (Nilges et al., 1988) with the program XPLOR (Brünger et al., 1986). For these calculations, nOe cross-peak intensities were categorized into three corresponding distance intervals. Although the effect of spin diffusion (which leads to overestimated interproton distances) is greatly attenuated in deuterated proteins (Arrowsmith et al., 1990; LeMaster, 1990; Reisman et al., 1991), a series of NOESY data collected with various mixing times was used in order to evaluate cross-peak intensity contributions arising from spin diffusion effects. Pseudoatoms were used for individual methyl groups and one nonstereospecifically resolved β-methylene proton pair and the interproton distances used for the structure calculations were adjusted accordingly (Wüthrich et al., 1983).

A total of 66 intra- and 55 interresidue nOe distance constraints were used in determining the bound CsA structure, and 77 intra- and 54 interresidue distances were used as constraints for the bound MeAla6-CsA structure. The backbone ϕ torsion angles were constrained for three residues (Abu2, Val5, and Ala7) by measuring the $^3J_{NH\alpha}$ coupling constants and evaluating the Karplus equation. All other ϕ dihedral angles, except for Sar3 and D-Ala8, were constrained to $-130° \pm 60°$ in order to maintain the proper Ramachandran geometries.

The starting structures for the calculations were generated by randomly assigning Cartesian coordinates to each atom. The first part of the calculations consisted of 20 cycles of 1-ps dynamics at 1000 K. At the beginning of each cycle the force constants were gradually increased. The repulsive force constants were initially set very low to allow atoms to approach and move through each other to improve the overall structure. The weakly coupled atoms were allowed to move independently in order to satisfy distance and geometrical constraints and to avoid folding problems. The next part of the calculations

Table I: Backbone Dihedral Angles for Free and Two CyP-Bound Cyclosporins

	CsA (free)		CsA (bound)		MeAla6-CsA (bound)	
residue	ϕ	ψ	ϕ	ψ	ϕ	ψ
MeBmt 1	−75.1	134.8	−69.5 ± 1.8	−179.8 ± 1.0	−104.8 ± 8.4	−180.7 ± 2.9
Abu 2	−145.9	124.0	−118.7 ± 6.3	71.8 ± 4.9	−118.4 ± 7.8	79.3 ± 4.1
Sar 3	56.3	−136.9	143.1 ± 6.7	−56.2 ± 5.0	156.7 ± 9.5	−63.7 ± 4.1
MeLeu 4	−111.7	52.6	−125.8 ± 3.8	99.0 ± 7.7	−117.5 ± 5.8	91.9 ± 10.6
Val 5	−134.2	162.4	−69.9 ± 4.6	153.1 ± 2.5	−68.3 ± 6.2	112.6 ± 1.2
MeLeu 6	−87.0	107.9	−122.5 ± 3.7	175.9 ± 5.6		
MeAla 6					−109.5 ± 5.8	173.4 ± 7.0
Ala 7	−91.7	64.0	−70.1 ± 5.7	127.0 ± 24.6	−64.3 ± 0.3	180.4 ± 0.2
D-Ala 8	87.1	−142.6	136.1 ± 30.0	−159.5 ± 1.3	92.0 ± 5.0	−158.6 ± 3.8
MeLeu 9	−116.8	113.4	−118.7 ± 3.0	70.4 ± 5.7	−125.8 ± 2.8	71.6 ± 9.2
MeLeu 10	−128.4	67.8	−125.5 ± 1.1	170.1 ± 3.1	−119.8 ± 3.1	169.2 ± 6.4
MeVal 11	−109.9	118.3	−124.1 ± 5.0	70.4 ± 4.8	−132.2 ± 5.2	79.9 ± 11.6

involved slowly cooling the molecule to 300 K in 25-deg steps with 150 fs of dynamics at each temperature. The energy constant for the repulsion terms was also gradually increased at each cycle to prevent further changes in the global conformation. The final part of the calculation involved all-atom Powell conjugate gradient energy minimization. No hydrogen-bond constraints were used in any part of the structure determinations.

Of the 100 structures determined for each drug, only those with all their ϕ and ψ backbone dihedral angles within one standard deviation of the mean were used in calculating the average structure (Table I). Planar ω torsion angles were maintained within 2°.

RESULTS

The dramatic simplification obtained from using fully deuterated CyP can be seen in Figure 1. Figure 1A shows a section of the NOESY spectrum obtained for the fully protonated CsA–CyP complex and contains both intra- and intermolecular dipolar information (from which interproton distance information can be determined). The corresponding region of the spectrum obtained for the complex formed with fully deuterated CyP (Figure 1B) shows only intramolecular interactions within CsA. Similar results to Figure 1B were obtained for MeAla6-CsA complexed to the deuterated receptor.

Interestingly, in the structures determined for free CsA both in the crystal (Petcher et al., 1976) and in organic solvents (Kessler et al., 1985) a cis bond exists between MeLeu9 and MeLeu10 (cis peptide bonds are characterized in NOESY spectra by a cross-peak between the corresponding α protons). However, in the receptor-bound structure, there were no cis peptide bonds present anywhere in either molecule. Due to the unusual peptide backbone of CsA (seven of the 11 residues are N-methylated), the sequential assignment of the drug was accomplished by noting that in trans peptide bonds involving *N*-methyl groups, the distance between the amide methyl protons and the α proton of the following residue is small, resulting in a NOESY cross-peak between these resonances.

The structure determined for CyP-bound CsA is markedly different from the previously reported structure of the free drug. Unlike the conformation of free CsA in organic solvents and in the crystal, in which the backbone is composed of a twisted β sheet and four intramolecular hydrogen bonds, the receptor-bound structure contains no discernible elements of regular secondary structure and no intramolecular hydrogen bonds. Also in sharp contrast to the structure of the free drug, in which six of the seven *N*-methyl groups are exposed to solvent, five *N*-methyl groups in the CyP-bound structure

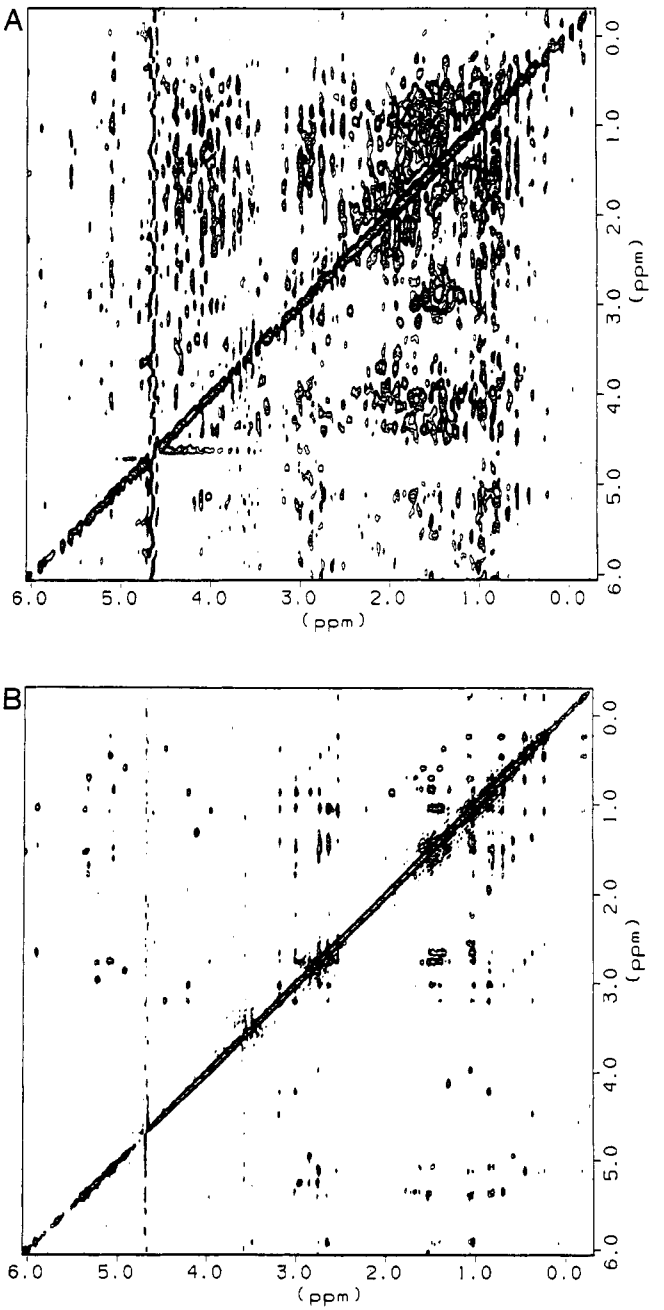

FIGURE 1: Portion of the NOESY spectra of CyP–CsA complexes (175-ms mixing time, 20 mM phosphate and 250 mM NaCl, pH 6.8, 25 °C). (A) Complex formed with protonated CyP, 0.75 mM complex, 0.5 mM dithiothreitol. (B) Complex formed with fully deuterated CyP, 0.4 mM complex, 0.2 mM Tris. Chemical shifts are referenced to HOD at 4.70 ppm.

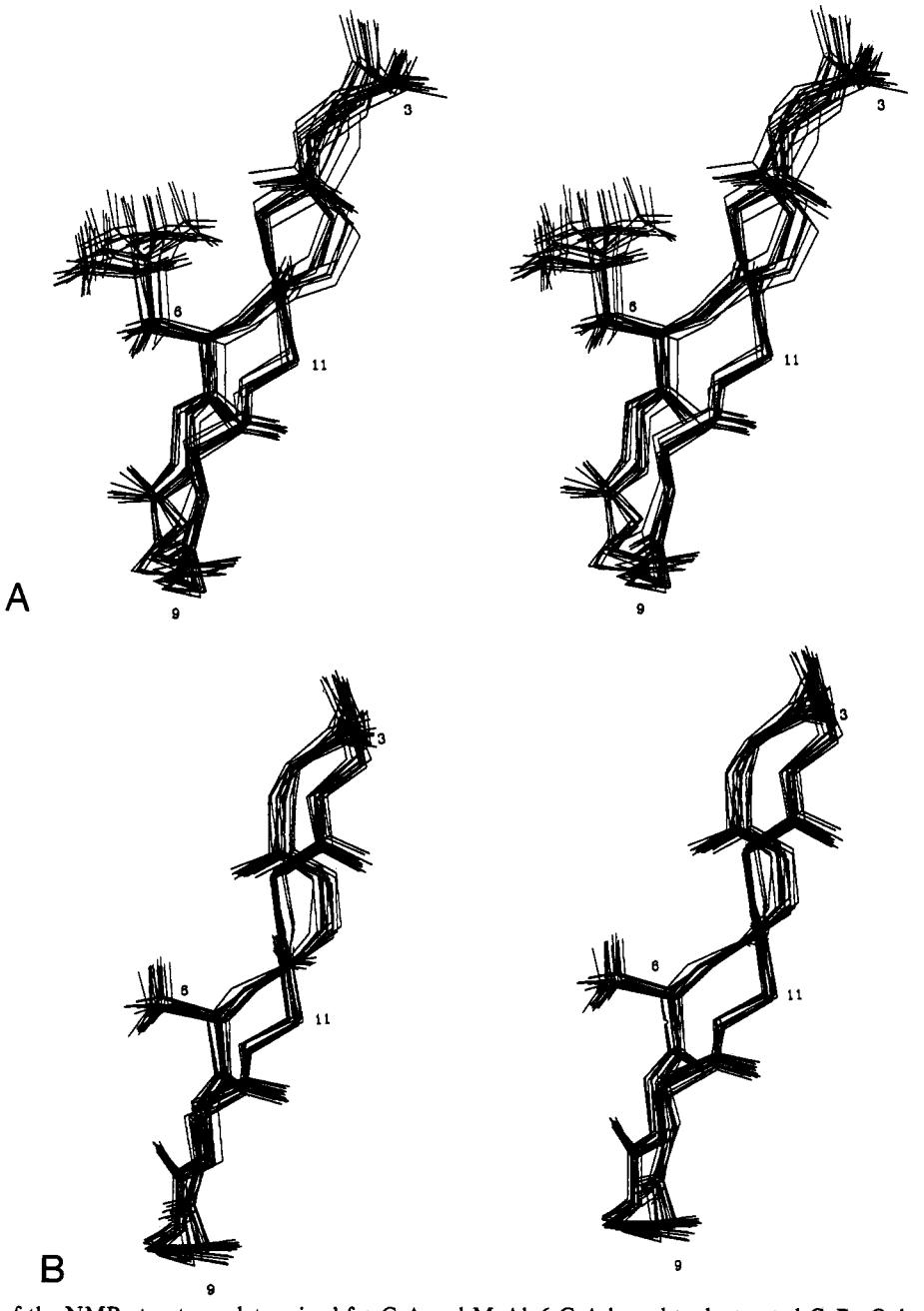

FIGURE 2: Stereoview of the NMR structures determined for CsA and MeAla6-CsA bound to deuterated CyP. Only the backbone atoms and the side chain of residue 6 are shown. (A) Superposition of the 16 best energy-minimized structures of CsA. (B) Superposition of the 17 best energy-minimized structures of MeAla6-CsA.

are oriented toward the center of the cyclic ring. The structure of the free MeAla-CsA analog, also determined in an organic solvent (Gooley et al., 1991), is very similar to the structure of free CsA. Likewise, the structure of CyP-bound MeAla6-CsA is very similar to that of CyP-bound CsA, as can be seen in Figure 2. The total rms deviation of the average backbones of the two bound drug structures is 0.54 Å. This rms difference is within the range of the deviation between crystal structure determinations of identical proteins in different crystal environments. The MeBmt1 residue adopts a more transannular orientation in the bound MeAla6-CsA structure as reflected in the observation of a nOe interaction between the MeBmt1 η proton and the MeAla6 β-methyl protons. As stated earlier, in the structures of both free drugs the peptide bond between residues 9 and 10 is in the cis orientation, and the same peptide bond is trans in the CyP-bound structures.

The structure determination of the bound drugs relied entirely, and exclusively, on the proton assignments of the drug resonances and the observed intramolecular nOes. Even though the amino acid side chains of CyP are fully deuterated and therefore "invisible" in all the high-resolution proton spectra collected, the exchangeable protein amide protons were observed in the spectra collected in 90% H_2O (10% D_2O was used for a lock signal). These protein amide resonances did not present any difficulty in the assignment of the four amide resonances from the drug since the CsA amides were the only amide protons that exhibited cross-peaks to their respective α protons in the fingerprint region of the scalar correlated experiments. Additionally, in the NOESY experiments, cross-peaks indicative of spatial proximity of these CyP amide protons to various protons of CsA allowed for the identification of those residues of CsA which are located at the binding site of CyP. Intermolecular nOes were observed between CyP and residues MeBmt1, Abu2, MeLeu9, MeLeu10, and MeVal11 of both CsA and MeAla6-CsA in the two complexes. On the basis of earlier NMR experiments on protonated CyP

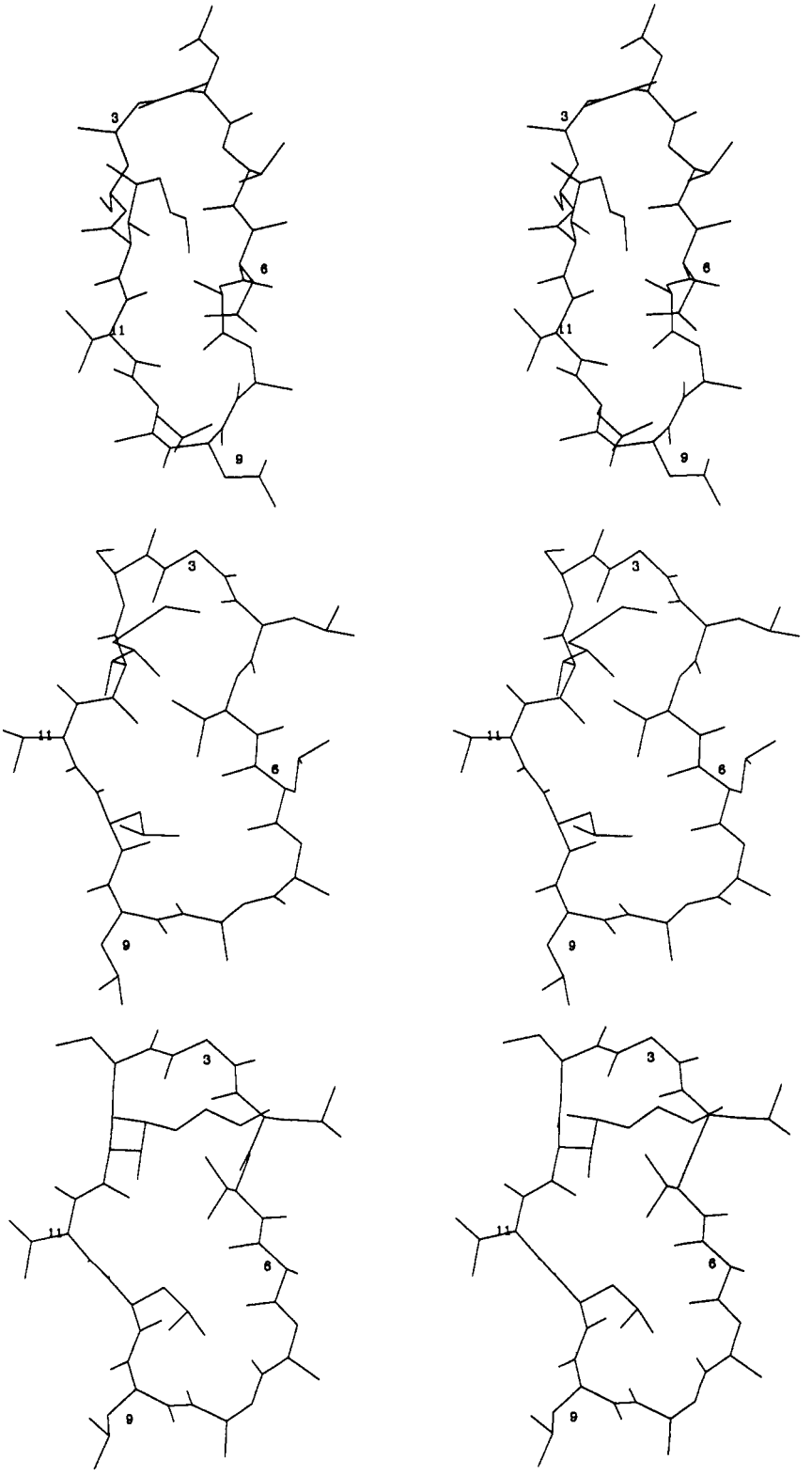

FIGURE 3: Comparison of various CsA structures. Protons are not shown for simplicity. (Top) Crystal structure of free CsA (Petcher et al., 1976). (Middle) Restrained energy-minimized average NMR structure of CyP-bound CsA determined from 16 individual structures. (Bottom) Restrained energy-minimized average NMR structure of CyP-bound MeAla6-CsA determined from 17 individual structures.

and its complex with CsA that were carried out in this laboratory (Dalgarno et al., 1986; Heald et al., 1990; Hsu et al., 1990), we were able to identify interactions between the indole ring proton of Trp121 of CyP and the δ-methyl protons of MeLeu9. No intermolecular nOes involving the six other residues of CsA were observed, which is in agreement with previous studies (Quesniaux et al., 1987) of CsA analogs which demonstrated that amino acid changes in positions 1, 2, 10, and 11 had the most profound effects on CyP binding. Although MeLeu9 was not implicated as being part of the "active surface", the observed intermolecular interaction of this residue supports the earlier prediction that the sole tryptophan residue in CyP is located in the binding site (Handschumacher et al., 1984; Heald et al., 1990).

DISCUSSION

The structures determined for both CsA and MeAla6-CsA bound to CyP, although almost identical, bear very little

similarity to their corresponding free structures determined by crystallography or by NMR in organic solvents (Figure 3). The difference between the free and bound structures can be envisaged as a complete ring inversion in which the cyclic peptide backbone of CsA has been turned "inside-out". It would appear that this incredible change in conformation was caused by binding to the receptor protein, CyP. However, a recent X-ray study of a CsA–Fab complex (Altschuh et al., 1992) suggests that the aqueous solution structure of free CsA is probably more similar to the bound structures than to the reported free structures. Unfortunately, due to the hydrophobic nature of CsA, it has not yet been possible to determine the conformation of the isolated drug in aqueous solution for direct comparison.

Since the immunosuppressive activity of many CsA drug analogs was correlated to their CyP binding affinity, it was believed that the binding surface of CsA somehow conveyed the immunosuppression activity. One noteworthy pair of exceptions involved amino acid changes at position 6. Substitution of MeLeu residue in CsA with MeAbu and MeAla did not substantially affect CyP binding, but the relative immunosuppressive activity decreased appreciably in the order MeLeu > MeAbu > MeAla (Durette et al., 1988). From our results pertaining to which CsA residues are directly involved in CyP binding, it is not surprising that changes in residue 6 do not affect binding. The fact that the backbone structures of CyP-bound CsA and MeAla6-CsA are very similar implies that the CsA-bound structure of CyP should not be different for the two drugs. Taken into account with the recent finding that the CsA–CyP complex binds to a complex involving calcineurin A and B and calmodulin in the presence of calcium (Liu et al., 1991), it is possible that the immunosuppressive activity is mediated by the solvent-exposed surface of the bound drug. This is corroborated by similar findings from studies on FK506 and its analogs and that the FK506–FKBP complex binds in the same pentapartite complex as the CsA–CyP complex (Liu et al., 1991). If this is the case and these drugs are composed of two different domains, binding and effector, a whole new perspective must be adopted for the design of new, more effective and less toxic immunosuppressive drugs. In the past, studies often concentrated on CsA analogs at positions 1, 2, 10, and 11 due to the cis peptide bond preceding MeLeu10 in the free drug and the identification of that region as the "active surface". However, it has since been shown that the "active surface" is actually the binding site and it appears that the solvent-exposed region centered about residue six serves an important structural role for the recognition of the CsA–CyP complex by other molecules involved in the immunosuppressive pathway.

Although similar information on the nature of the intermolecular interactions and the stereospecific assignment of the δ-methyl groups of Val5 and MeLeu10 were determined from the isotope-filtered experiments (Fesik et al., 1991; Weber et al., 1991), it is reassuring that the final structures determined for CyP-bound CsA were the same regardless of the strategy employed. In the past, random fractional and selective deuteration has been used primarily to improve spectra quality, interpretation, and quantification (Brodin et al., 1989; Arrowsmith et al., 1990; LeMaster, 1990; Reisman et al., 1991). However, the use of completely deuterated recombinant proteins has not been fully exploited to study macromolecular complexes and structures in solution (Seeholzer et al., 1986). Indeed, there are several distinct advantages to the homonuclear approach using fully deuterated receptor proteins as we have described. The ^{13}C-labeled medium necessary for producing the isotopically labeled drug used in the heteronuclear experiments is more expensive than ^{2}H-labeled medium. Biosynthetic labeling can only be efficiently used for naturally occurring CsA, and therefore determining the structure of bound CsA analogs would require the synthetic chemical generation of labeled analogs. The use of fully deuterated receptor proteins does not require specialized multinuclear probes, any of a number of CsA analogs could be studied, and data collection took only 2 days compared to the 9–10 consecutive days required for the heteronuclear 3D strategy (Fesik et al., 1991; Weber et al., 1991). One may consider the integration of these two methods for studies on even larger proteins and biomolecular complexes. Complete deuteration techniques can also be applied to NMR studies of protein–nucleic acid interactions and enzymatic investigations.

With all the new information about the CsA–CyP complex and its interactions and all the interest in designing immunosuppressive CsA analogs which exhibit reduced toxicity and fewer undesirable side effects, this complex may serve as a model for developing future strategies in the field of rational drug design. It is clear that if one wishes to invoke structure–activity arguments in drug design, a comparison of the relevant free and receptor-bound conformations of both active and nonactive analogs is required for elucidating key structural features pertinent to drug binding and activity.

ACKNOWLEDGMENT

We thank the Martek Corp. (Columbia, MD) for the generous gift of the deuterated media, Professors S. Schreiber and C. Walsh for the plasmid construct, and Professor R. E. Handschumacher for the cyclosporins and helpful discussions.

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