Volume 300, number 3, 291-300 FEBS 10899 April 1992

Cyclosporin A–cyclophilin complex formation

A model based on X-ray and NMR data

Claus Spitzfaden[a], Hans-Peter Weber[b], Werner Braun[a], Joerg Kallen[b], Gerhard Wider[a], Hans Widmer[b], Malcolm D. Walkinshaw[b] and Kurt Wüthrich[a]

[a]*Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule-Hönggerberg, CH-8093 Zürich, Switzerland and* [b]*Preclinical Research, Sandoz Pharma Ltd., CH-4002 Basel, Switzerland*

Received 20 February 1992

The previously determined 3D NMR solution structure of cyclophilin-bound cyclosporin A (CsA) was docked onto the X-ray crystal structure of cyclophilin. Intermolecular nuclear Overhauser effects (NOE) between CsA and cyclophilin were used as constraints in a restrained energy minimization to generate a model of the complex which satisfied all the NOE distance constraints. The model shows that the residues 9 to 11 and 1 to 5 of the cyclic CsA molecule are in contact with cyclophilin. Comparing the model of the CsA–cyclophilin complex to the X-ray crystal structure of a complex of cyclophilin with a substrate for peptidyl-proline *cis-trans* isomerase activity, i.e. the linear tetrapeptide substrate ac-Ala-Ala-Pro-Ala-amc (ac, acetyl; amc, amidomethylcoumarin), one notices that the contacting peptide segments in the two ligands are oriented in opposite directions, and that the side chain of MeVal-11 of CsA superposes rather precisely with the position of the prolyl residue in ac-Ala-Ala-Pro-Ala-amc.

Cyclosporin A; Cyclophilin; Complex formation; NMR structure

1. INTRODUCTION

Cyclosporin A (CsA) is a widely used, effective immunosuppressive drug which has revolutionized the field of clinical organ transplantation by significantly improving the survival of kidney, liver and heart allografts [1]. The biological mechanism of CsA action involves the prevention of T-cell activation in response to antigen recognition. One of the important events in T-cell activation is the production of lymphokines, including interleukin-2. CsA blocks the signal transduction pathway in T-cells and prevents the transcription of interleukin-2. There are a large number of proteins involved in the transduction pathway and details of the role played by CsA are only now beginning to emerge.

The major intracellular receptor for CsA is the protein cyclophilin. This protein has peptidyl-prolyl *cis-trans* isomerase (PPIase) activity and has been shown to enhance the rate of refolding of some proteins after denaturation [2]. Blocking of the *cis-trans* isomerase activity is, however, not sufficient to induce immunosuppression [3]. This fits with the observation that the intracellular concentration of cyclophilin is greater than the concentration of CsA required for immunosuppression. It is known that a complex of CsA bound to cyclophilin binds to and inhibits the serine-phosphatase activity of calcineurin, which could play a crucial role in the signal transduction pathway [4].

There is an intriguing overlap of biological activity between cyclosporin A and the chemically unrelated macrolide FK506. Both CsA and FK506 show immunosuppression by blocking the production of interleukin-2. The specific receptor for FK506 is the FK-binding protein (FKBP), which consists of 107 amino acids and also shows PPIase activity [5,6]. Furthermore, the FK506–FKBP complex inhibits the serine-phosphatase activity of calcineurin competitively with the CsA–cyclophilin complex [4].

Considerable efforts in many laboratories have been made to extract information on the 3D structure of immunophilins and their ligands in an attempt to improve our understanding of the biological and enzymatic activity. The structure of FKBP has been solved in solution by NMR [7,8], and that of the FKBP–FK506 complex by X-ray crystallography [9]. X-ray crystal structures of cyclophilin complexed with the tetrapeptide PPIase substrate ac-Ala-Ala-Pro-Ala-amc (ac, acetyl; amc, amidomethylcoumarin) [10] and without substrate [11] have also been published. Complete sequence-specific NMR assignments for the polypeptide backbone and for part of the amino acid side chains have been obtained for free cyclophilin and for cyclophilin in the 1:1 complex with CsA, using 2D and 3D NMR methods [10,12,13]; chemical shift differences between the backbone resonances of the free and the complexed form of the protein (see the Appendix) gave initial indications on the CsA binding site, which appears

Correspondence address: K. Wüthrich, Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, 8093 Zürich, Switzerland. Fax: (41) (1) 371 4873.

to be in the same general area of the protein surface as the binding site of the tetrapeptide substrate for PPIase activity of cyclophilin [10,13].

The NMR structure of CsA bound to cyclophilin has also been determined [14,15], which showed that the conformation of bound CsA is fundamentally different from both the NMR structure of free CsA determined in chloroform [16,17] and the X-ray crystal structure of CsA [16]. An X-ray structure of CsA bound to cyclophilin has not yet been determined; all crystal forms of the cyclophilin–CsA complex yet obtained have multiple copies of the complex in the asymmetric unit [18]. In this paper we present a model of the CsA– cyclophilin complex based on the refined X-ray crystal structure of cyclophilin in the complex with ac-Ala-Ala- Pro-Ala-amc, the NMR solution structure of CsA bound to cyclophilin, and a set of intermolecular nuclear Overhauser effects (NOEs) measured in solution. The structure of this complex is of considerable interest as a starting point for the design of novel immunosuppressant drugs.

2. MATERIALS AND METHODS

2.1. *Assignment of intermolecular NOEs in the cyclophilin–CsA complex*

The production of uniformly ^{15}N- or ^{13}C-labeled CsA and the preparation of 1:1 complexes of these isotope-labeled species with cyclophilin were described elsewhere [14]. Sequence-specific NMR assignments were previously obtained for cyclophilin-bound CsA [14,15] as well as for the polypeptide backbone and part of the side chain protons of free cyclophilin and cyclophilin in the CsA complex [10,12,13] (see also the Appendix). On this basis, intermolecular NOEs between CsA labeled with ^{13}C or ^{15}N and unlabeled cyclophilin, or between unlabeled CsA and ^{15}N-labeled cyclophilin were investigated.

The largest number of intermolecular NOEs were collected from a complex of uniformly ^{13}C-labeled CsA and unlabeled cyclophilin, using 3D ^{13}C-correlated [^{1}H,^{1}H]-NOESY and 2D [^{1}H,^{1}H]-NOESY with a ^{13}C(ω_1,ω_2)-double-half-filter [19–21]. Additional NOEs were identified in 2D [^{1}H,^{1}H]-NOESY spectra recorded with a ^{15}N(ω_2)-half-filter for the complex of ^{15}N-labeled CsA with unlabeled cyclophilin, and in a 3D ^{15}N-correlated [^{1}H,^{1}H]-NOESY spectrum of uniformly ^{15}N-labeled cyclophilin complexed with unlabeled CsA. These NMR experiments were recorded at a ^{1}H-frequency of 500 or 600 MHz using Bruker AM500 or AMX600 spectrometers.

2.2. *Docking of CsA against the cyclophilin surface*

A starting model for the CsA–cyclophilin complex was generated using the X-ray crystal structure coordinates of cyclophilin in the complex with ac-Ala-Ala-Pro-Ala-amc refined at 2.3 Å resolution [22], and the coordinates of the CsA conformer with the lowest conformational energy (99.6 kcal/mol) among the 120 AMBCOM-minimized [23] conformers used to represent the solution structure of cyclophilin-bound CsA [14]. Using the interactive graphics system MidasPlus [24], the CsA structure was docked manually onto the cyclophilin structure, whereby the structures of the two components were kept unchanged. In this procedure an initial set of intermolecular NOE contacts identified in the heteronuclear-edited ^{1}H NMR spectra was monitored. The acceptability of this starting structure was evaluated by checks for violations of NOE distance constraints and short van der Waals contacts. After this initial docking the structure of the complex was searched for additional short ^{1}H–^{1}H distances between cyclophilin and CsA, and the NMR spectra were checked for the presence of corresponding NOESY cross peaks. Finally, to obtain the input for a restrained refinement of the docked structure with the molecular mechanics and graphics package Insight/Discover [25], all the assigned intermolecular NOEs were added to the intramolecular NOEs and the dihedral angle constraints that had been used to determine the conformation of cyclophilin-bound CsA [14].

The following energy minimization procedure was used for the refinement of the CsA–cyclophilin complex: a shell of cyclophilin residues containing at least one atom closer than 9.0 Å from the nearest CsA atom was identified, i.e. the cyclophilin residues 54–77, 81, 82, 92, 97–128, and 147–152. For the amino acid residues inside this shell only the Cα atoms were held fixed during the refinement, with the exception of residues 72–75 and 102–104, which were completely free to move. (For these residues we observed that backbone proton to backbone proton NOE intensities in the ^{15}N-correlated [^{1}H,^{1}H]-NOESY spectrum of the complex were different from those in the free protein.) All cyclophilin atoms outside of this shell were held fixed, and all atoms of CsA were free to move during the refinement. Both the intramolecular NOE distance constraints in CsA [14] and the intermolecular NOE constraints were applied in the minimization with a harmonic potential at the upper and lower distance limits. The maximum force in the constraints was limited to 600 kcal/Å at a deviation of 0.2 Å from the limits. The lower limit was uniformly set to 2.2 Å.

3. RESULTS

Fig. 1 shows a 2D [^{1}H,^{1}H]-NOESY spectrum of a complex of uniformly ^{13}C-labeled CsA and unlabeled cyclophilin in H_2O solution recorded with a ^{13}C(ω_1,ω_2)-double-half-filter [19–21]. Previously, the ^{13}C(ω_1,ω_2)-doubly-selected subspectrum from a similar experiment recorded in D_2O solution was used to collect the input data for the computation of the conformation of cyclophilin-bound CsA [14]. Here the ^{13}C(ω_2)-selected / ^{13}C(ω_1)-filtered subspectrum (Fig. 1) was used to observe NOE cross peaks with ω_1-frequencies of protons that are not bound to ^{13}C (i.e. all cyclophilin protons and the amide protons of CsA) and ω_2-frequencies of ^{13}C-bound protons. In a D_2O solution of the complex, more than 70 NOEs were thus observed, involving protons of the residues 9, 10, 11, 1, 2, 3, 4 and 5 of CsA, and aliphatic or aromatic protons of cyclophilin. The spectrum recorded in H_2O solution (Fig. 1) contained further 11 intermolecular NOEs with labile amide protons of cyclophilin. Additional NOEs were observed with the other NMR experiments mentioned in the preceding section, which used either ^{15}N-labeled CsA bound to unlabeled cyclophilin, or unlabeled CsA bound to ^{15}N- labeled cyclophilin.

For cyclophilin, complete sequence-specific NMR assignments are available for the polypeptide backbone [10,12,13] (see Appendix), but assignments have so far been elucidated only for a limited selection of amino acid side chains. Therefore, although all intermolecular NOESY cross peaks could be attributed to interactions with specified individual CsA protons, the corresponding cyclophilin protons could be identified only for part of the intermolecular NOESY cross peaks. In the presentation of Table I, three categories of NOE distance constraints have been distinguished. A first group in-

cludes 16 NOEs that were unambiguously assigned using the known ^{1}H chemical shifts of cyclophilin-bound CsA [14] and of CsA-bound cyclophilin. For the latter the backbone assignments [10,12] are listed in the Appendix, and assignments for the β protons of Ala-101 and Ala-103, and for the δ- and ε-protons of Trp-121 were obtained from [^{1}H,^{1}H]-TOCSY spectra. These 16 NOEs were used for the initial docking of CsA onto cyclophilin. A second group comprising the four NOEs 4, 10, 12 and 20 (Table I) were identified on the same basis during re-examination of the spectra after the initial docking. The third group contains 12 NOEs of individually assigned CsA protons with the α-proton of Ala-103 and side-chain protons of Arg-55, Ile-57, Leu-122 and His-126 in cyclophilin. These cyclophilin resonances had not been assigned to individual protons using the spectral data alone. Once a model of the complex was available from the initial docking (see below), assignments were derived from reference to this molecular structure. In particular, since the αH resonance of Ala-103 is nearly degenerate with those of Ala-101 and Asn-102 (see Appendix) the assignment of the NOE 8 (Table I) was only accepted after an inspection of the initial model showed that the other likely assignments could be excluded because of the long distances to ηCH_3 of MeBmt-1. The δ-methyl group of Ile-57 is the only methyl group (besides γCH_3 of Ile-57, which was already assigned to NOE 20) in close proximity of $\delta^2 CH_3$ of MeLeu-9 in CsA (NOE 21 in Table I). The NOE with $\gamma^1 CH_3$ of Val-5 (NOE14 in Table I) could be assigned to an εNH group of Arg from the unique ^{15}N chemical shift, but only the inspection of the structure of the complex showed that the Arg spin system in position 55 is involved in the NOE. [^{1}H,^{1}H]-TOCSY connectivities had shown that the NOEs 23, 24, 26, 27, 31 and 32 all involved the same isopropyl group of cyclophilin, and inspection of the model then implied that it had to be Leu-122. Finally, the two strong NOEs 6 and 28 and the weak NOE 3 with a non-exchanging proton in the aromatic frequency range were assigned to His-126 because this was the only aromatic group in close proximity to the CsA protons in question.

For the initial docking, interactively monitored rigid-body movements of the CsA structure relative to cyclophilin were guided by the 16 intermolecular NOE contacts 1, 2, 5, 7, 9, 11, 13, 15–19, 22, 25, 29 and 30 in Table I. In the resulting initial model, a possible binding groove was identified, consisting on one side of the hydrophobic residues Phe-60, Met-61, Phe-113, Trp-121 and Leu-122, and on the other side of polar confines formed by the polypeptide backbone segment -Met^{100}-Ala-Asn-Ala-Gly^{104}-, and the side chains of Arg-55 and Gln-63. The 16 intermolecular NOEs can be classified into five classes linking, respectively, Bmt-1 of CsA with the segment 101 to 103 of cyclophilin, γCH_3 of Abu-2 with the residues Ala-101 and Ala-103, the *N*-methyl group of MeLeu-4 with the methyl group of Ala-103,

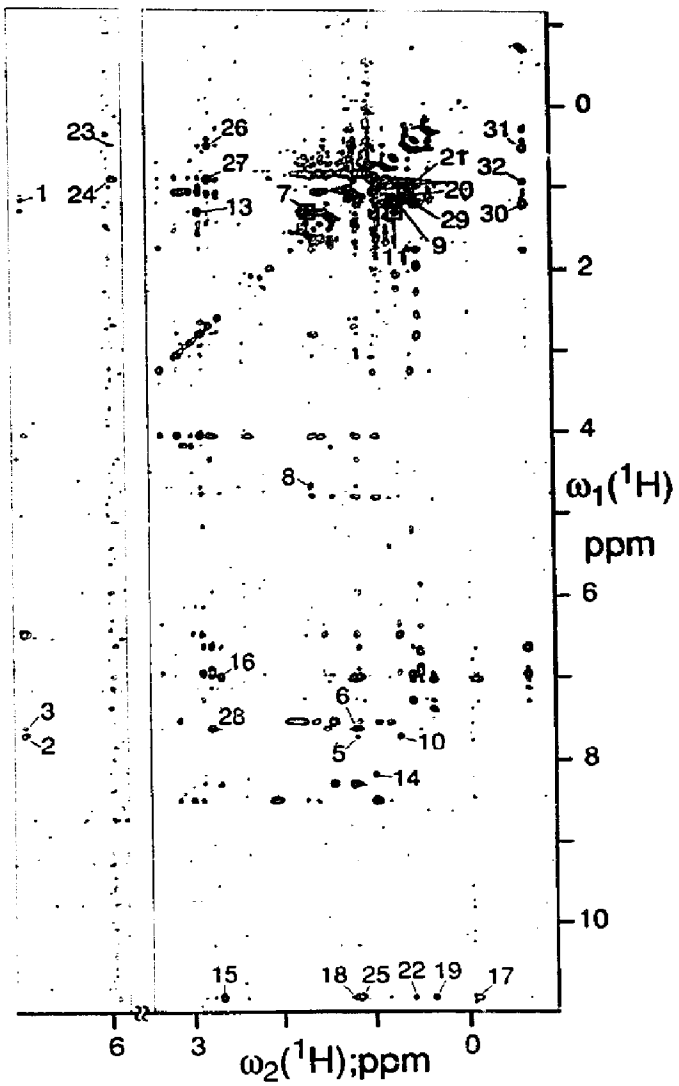


Fig. 1. Spectral regions (ω_1 = −1 to 11 ppm, ω_2 = −1 to 3.4 ppm and 5.8 to 6.9 ppm) of a [^{1}H,^{1}H]-NOESY spectrum of the complex formed by unlabeled cyclophilin and uniformly ^{13}C-labeled CsA recorded with a $^{13}C(\omega_1,\omega_2)$-double-half-filter. The $^{13}C(\omega_2)$-selected-$^{13}C(\omega_1)$-filtered subspectrum is shown (mixing time 80 ms, proton frequency 500 MHz, measuring time 180 h, complex concentration 2 mM, solvent 90% H_2O/10% D_2O, temperature 23°C, pH 6.0). All NOEs in the spectral regions shown that have been used for the structure refinement (see text) are identified by numbers which represent the code for the entries in Table I.

MeLeu-9 and MeLeu-10 with the Trp-121 indole ring, and both γ-methyl groups of MeVal-11 with the methyl group of Ala-101. Due to the wide distribution of the NOE contacts over the binding site of cyclophilin (Fig. 2A,B) the experimental intermolecular contacts determined a unique orientation of CsA relative to the cyclophilin molecule. A check of this starting model for short ^{1}H ^{1}H contacts, using the same atomic radii for the atom types as in distance geometry calculations with the program DISMAN [27], showed that all intermolecular contacts could be fulfilled within an upper limit of 5.0

Table 1

Intermolecular NOE distance constraints used for the modeling of the cyclophilin-CsA complex

NOE[a]	CsA	δ (ppm)	Cyclophylin	δ (ppm)	Intensity[b]	Limit (Å)[c]	Distance (Å)[c]
1	MeBmt-1 αH	6.82	Ala-101 βCH$_3$	1.21	w	6.0	3.68
2	MeBmt-1 αH	6.82	Asn-102 NH	7.69	m	5.0	4.68
(3)	MeBmt-1 αH	6.82	His-126 εH[d]	7.62[d]	w	7.4[d]	5.67[d]
4*	MeBmt-1 βH	4.05	Asn-102 NH	7.69	w	5.0	3.82
5	MeBmt-1 δCH$_3$	1.14	Asn-102 NH	7.69	m	6.0	5.22
(6)	MeBmt-1 δCH$_3$	1.14	His-126 εH[d]	7.62[d]	s	7.4[d]	4.80[d]
7	MeBmt-1 ηCH$_3$	1.62	Ala-103 βCH$_3$	1.35	s	6.0	6.01
(8)	MeBmt-1 ηCH$_3$	1.62	Ala-103 αH	4.67	s	5.0	5.01
9	Abu-2 γCH$_3$	0.68	Ala-101 βCH$_3$	1.21	s	6.0	3.57
10*	Abu-2 γCH$_3$	0.68	Asn-102 NH	7.69	m	6.0	5.14
11	Abu-2 γCH$_3$	0.68	Ala-103 βCH$_3$	1.35	m	7.0	5.62
12*	Abu-2 NH	6.42	Asn-102 NH	7.69	w	6.0	5.76
13	MeLeu-4 NCH$_3$	2.84	Ala-103 βCH$_3$	1.35	s	6.0	3.77
(14)	Val-5 γ'CH$_3$	0.95	Arg-55 εNH	8.17	m	6.0	3.02
15	MeLeu-9 NCH$_3$	2.64	Trp-121 εNH	10.90	s	5.0	4.23
16	MeLeu-9 NCH$_3$	2.64	Trp-121 δH	7.01	s	5.0	4.79
17,18	MeLeu-9 βH	-0.12/1.17	Trp-121 εNH	10.84	m,m	5.0, 5.0	2.76, 2.92
19	MeLeu-9 δ'CH$_3$	0.32	Trp-121 εNH	10.84	m	6.0	5.05
20*	MeLeu-9 δ'CH$_3$	0.54	Ile-57 γCH$_3$	1.33	s	6.0	4.18
(21)	MeLeu-9 δ'CH$_3$	0.54	Ile-57 δCH$_3$	1.22	m	7.0	4.85
22	MeLeu-9 δ'CH$_3$	0.54	Trp-121 εNH	10.84	w	6.0	6.00
(23)(24)	MeLeu-10 αH	6.00	Leu-122 δ'/δ" CH$_3$	0.55/0.96	m,m	6.0, 6.0	4.90, 3.86
25	MeLeu-10 δ'CH$_3$	1.12	Trp-121 εNH	10.84	m	6.0	5.21
(26)(27)	MeVal-11 NCH$_3$	2.74	Leu-122 δ'/δ" CH$_3$	0.55/0.96	s,s	6.0, 6.0	4.21, 3.22
(28)	MeVal-11 NCH$_3$	2.74	His-126 εH[d]	7.62[d]	s	7.40[d]	4.41[d]
29	MeVal-11 γ'CH$_3$	0.46	Ala-101 βCH$_3$	1.21	s	6.0	4.43
30	MeVal-11 γ"CH$_3$	-0.69	Ala-101 βCH$_3$	1.21	m	7.0	7.00
(31)(32)	MeVal-11 γ"CH$_3$	-0.69	Leu-122 δ'/δ" CH$_3$	0.55/0.96	s,s	6.0, 6.0	3.32, 5.36

[a] The numbers correspond to those used in Fig. 1 to identify the locations of the corresponding NOESY cross peaks. Three groups of NOEs are distinguished (see text): The 16 NOEs identified by numbers only were assigned based on sequence-specific resonance assignments in CsA and cyclophilin, and were used for the initial docking, those identified by numbers with an asterisk were assigned later-on on the same basis, and those identified by numbers in parentheses were assigned based on sequence-specific resonance assignments in CsA and inspection of the molecular model obtained from the initial docking of CsA onto cyclophilin (see text for details).

[b] s, strong; m, medium; w, weak.

[c] These two columns list the upper distance limits derived from the observed NOEs (s ≤ 4.0 Å, m ≤ 5.0 Å, w ≤ 5.0 Å) and the corresponding distances in the structure of the complex, respectively (pseudoatom corrections [26] were added to constraints involving methyl groups (1.0 Å per methyl group) and imidazole ring protons (2.4 Å).

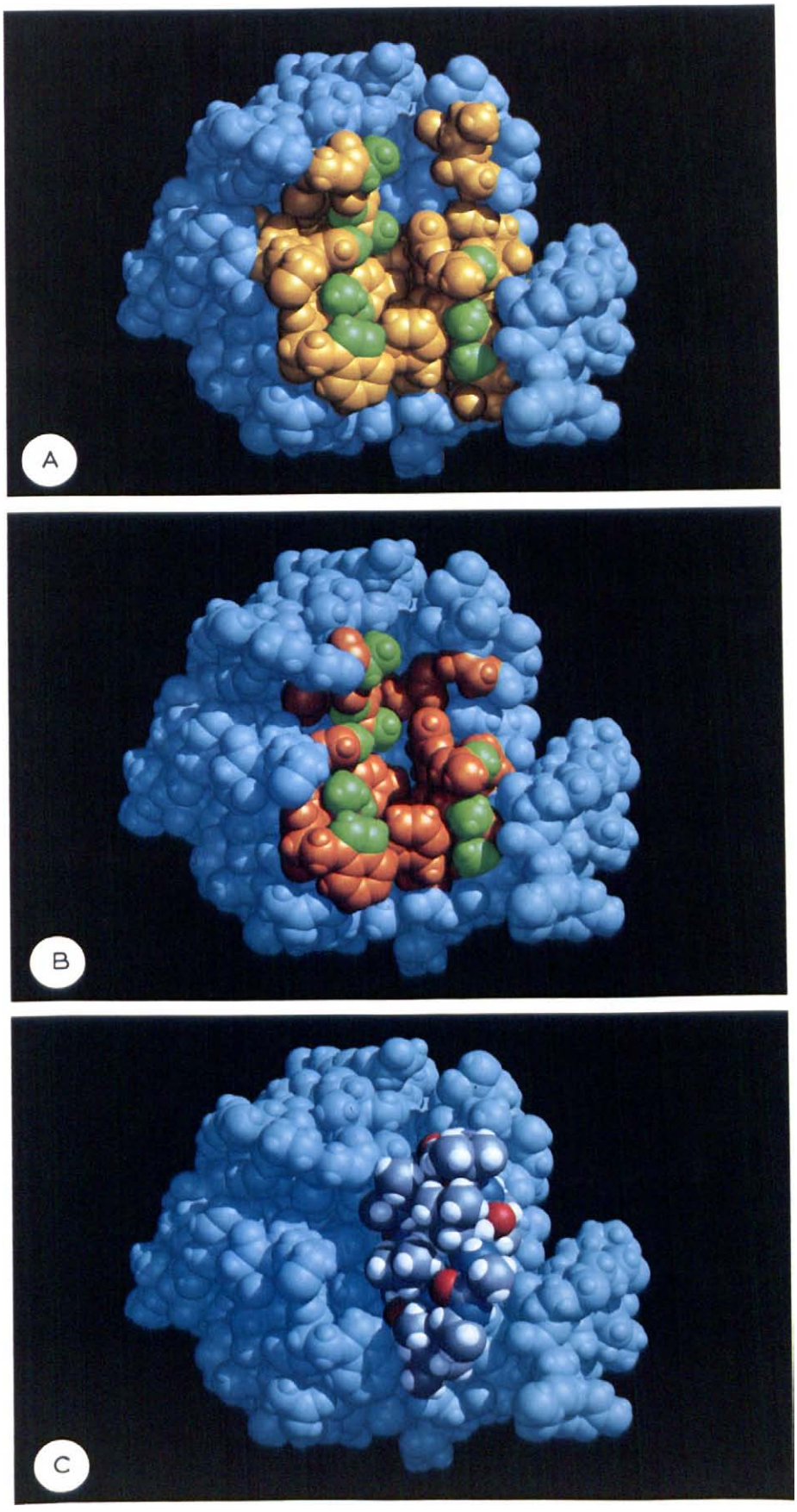
[d] Based on the characteristic chemical shift [26] the imidazole ring resonance at 7.62 ppm was tentatively assigned to the ε proton. For the structure calculation, however, the NOE distance constraints 3, 6 and 28 were referred to the center of the imidazole ring, using an appropriate pseudoatom correction (see [c] above).

Å. Among all the van der Waals contacts there was only one violation greater than 0.2 Å, i.e. 0.36 Å. An additional short contact between the hydroxyl group of MeBmt-1 in CsA and the carbonyl oxygen of Asn-102 in cyclophilin was accepted at this stage because it could be an indication of possible hydrogen bond formation.

Restrained energy minimization of the structure of the complex using DISCOVER [25] resulted in a molecular model (Figs. 2 and 3) that satisfied all intra- and inter-molecular NOE constraints (Table 1) and contained no unacceptably large van der Waals violations. 2000 iterations of energy minimization reduced the con-

→

Fig. 2. Intermolecular interactions in the cyclophilin-CsA complex obtained as the result of restrained energy minimization, visualized with space filling molecular models. (A) Binding site of CsA on the cyclophilin surface. Cyclophilin is shown in light blue, except that those residues with significant backbone chemical shift variations between free and CsA-bound cyclophilin (Appendix) are yellow, and atom groups involved in assigned intermolecular NOE contacts (Table 1) are green. Chemical shift differences for NH greater than 0.10 ppm, for αH greater than 0.05 ppm and for ^{15}N greater than 0.5 ppm were considered to be significant [10]. (B) Same as (A), except that brown color indicates all those residues with intermolecular contacts between heavy atoms of less than 3.6 Å. (C) CsA bound to cyclophilin. CsA is colored according to the atom types: white, hydrogen; grey, carbon; blue, nitrogen; red, oxygen. In the orientation used, Trp-121 is in the lower left edge of the yellow area in (A), and the binding groove runs vertically through the center of the molecule. The color illustrations were prepared with the program Midas Plus [24].


A
B
C

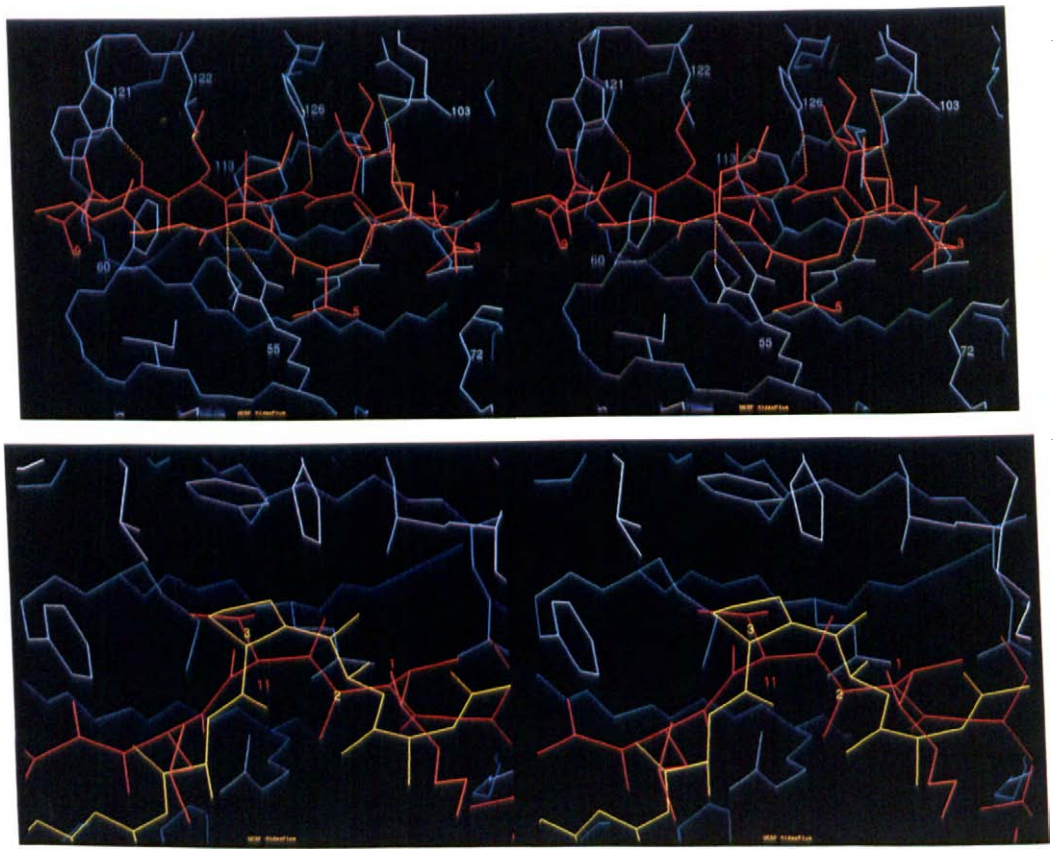


Fig. 3. (A) Stereo view of the binding site in the cyclophilin-CsA complex. Color code: blue for cyclophilin, red for CsA. For cyclophilin only the backbone atoms and the heavy atoms of the contact residues are shown, for CsA all heavy atoms are shown. In addition, intermolecular hydrogen bonds are indicated by dashed lines and the hydrogen atoms involved in these hydrogen bonds are also shown. (B) Comparison of the proposed location of CsA (red) and the experimentally determined [22] location of ac-Ala-Ala-Pro-Ala-ome (yellow) in the complex with cyclophilin (light-blue). The atom coordinates were taken from the present work and from the crystal structure determination [22], respectively, and the two complexes were superimposed for minimal RMSD of the backbone heavy atoms of residues 1–165 of cyclophilin. In (A), selected residues of cyclophilin and CsA are identified with the sequence [illegible]. In (B) the residues 2 and 3 of ac-Ala-Ala-Pro-Ala-ome and the spatially corresponding residues MeVal-11 and MeBmt-1 of CsA are identified by numbers positioned near the α carbon atoms. The color illustrations were prepared with the program Midas Plus [24].

straints energy from an initial value of 152 kcal/mol to 5.9 kcal/mol, and the final upper limit violations converged to 0.002 Å on average, with a maximal deviation of 0.017 Å. All dihedral angle constraints were satisfied to within better than 1°. The changes in positions of the atoms of cyclophilin that were allowed to move during the refinement were rather small, and sizeable displacements occurred mainly in some long external side chains far away from CsA: the RMS change for all these atoms was 0.64 Å. The largest individual displacements were found near residue Asn-102, where the backbone carbonyl oxygen was forced to move by about 1.57 Å to accommodate the MeBmt-1 hydroxyl group. Smaller, yet significant displacements were observed for the backbone atoms of the residues Asn-71 to Gly-75. Between the starting and final conformations of the docked CsA, an all heavy-atom RMS fit of 0.9 Å was found. The largest atom displacements between the two conformations were observed at MeLeu-4, and are probably caused by the requirement that the *N*-methyl group of this residue must satisfy a strong intermolecular NOE to Ala-103 βCH_3.

Table II

Intermolecular hydrogen bonds implicated by the presently described model of the cyclophilin-CsA complex (see also Fig. 3A).

Atoms involved in the hydrogen bond		
CsA	Cyclophilin	d (Å)*
MeBmt-2 γ OH	Asn-102 CO	2.83
MeBmt-1 CO	Gln-63 εNH	3.08
MeLeu-9 CO	Trp-121 εNH	2.96
MeLeu-10 CO	Arg-55 ηNH	3.03
MeVal-11 CO	His-126 εNH	3.01

*The distance from the heavy atom of the donor group to the acceptor atom is given.

To test the stability of the resulting molecular complex, further energy refinements were performed without any NMR constraints. In a first energy minimization of the complex performed in vacuo, the non-contacting CsA residues 6–8 were bent by about 1 to 2 Å

toward the cyclophilin surface, while the other residues remained close to their original positions. This clearly indicated that the cyclophilin-bound CsA conformation is unstable in vacuo. Therefore, a second energy refinement was performed, again without NMR constraints but with a 15 Å thick shell of water around the CsA and the neighbouring residues of cyclophilin. After 2000 iterations of energy refinement with these conditions, the CsA molecule had moved only very slightly away from its starting position. The average movement of the backbone atoms of CsA was only 0.26 Å, the average change for all atoms 0.37 Å, and the changes for the moving cyclophilin atoms were equally small.

4. DISCUSSION

The refined model of CsA docked onto cyclophilin was used to define a binding site, whereby cyclophilin residues having one or more heavy atoms within 3.8 Å of a heavy atom of CsA were regarded as part of the contact surface. With this criterion, thirteen contact residues were found, i.e. Arg-55, Phe-60, Met-61, Gln- 63, Gly-72, Ala-101, Asn-102, Ala-103, Gln-111, Phe- 113, Trp-121, Leu-122 and His-126. Using the same criterion, one finds eleven contact residues in the previously described crystal structure of the cyclophilin complex with ac-Ala-Ala-Pro-Ala-amc [22], i.e. Arg-55, Ile- 57, Phe-60, Gln-63, Ala-101, Asn-102, Gln-111, Phe- 113, Leu-122, His-126 and Arg-148. The fact that there are nine identical contact residues in the two complexes clearly shows that the binding site for CsA overlaps with the PPIase active site.

The thirteen residues in contact with the docked CsA molecule (Fig. 2B) are part of an active-site groove which rests on three of the antiparallel strands of the eight-stranded β barrel, involving residues Phe-60, Met-61 and Gln-63, Phe-113 and Gln-111, and Arg-55, respectively. Three other groups of residues, i.e. Trp- 121, Leu-122 and His-126, Ala-101, Asn-102 and Ala-103, and Gly-72 are located in three separate loop regions which protrude from the surface of the barrel by some 10 to 15 Å (Fig. 2). With regard to future, similar applications of the NMR technique it is interesting to note that the contact area of cyclophilin is well within the surface region made up of residues with sizeable chemical shift differences of the backbone atoms between free and CsA-bound cyclophilin (Figs. 2A,B), and that chemical shift effects are seen well beyond the direct contact area.

The docking of CsA to cyclophilin is reminiscent of a coin going part-way into a slot-machine; only one rim of the circular CsA molecule, formed by the residues 9, 10, 11, 1, 2 and 3, sticks in the slot (Fig. 2C). The isopropyl group of Val-5 on the surface of the complex makes close contact with Arg-55 (see also Table I). The complementarity of the fit into the active site groove is very good, with an estimated cyclophilin contact surface area of 320 Å^2 [28]. This is somewhat larger than the contact surface area of 230 Å^2 between cyclophilin and ac-Ala-Ala-Pro-Ala-amc measured in the crystal structure [22]. The close contacts with CsA involve some 26 atoms from the 13-residue binding site of cyclophilin. The side chain of MeVal-11 fits snugly into the deep 'proline-binding pocket' formed by Phe-60, Met-61, Phe-113 and Leu-122 [22]. The MeBmt-1 and MeLeu-9 side chains form good hydrophobic contacts with two of the protruding active site loops, and Abu-2 guards the entrance to an unfilled cleft which is a likely location for an extended protein substrate [22]. The present model of the cyclophilin–CsA complex is well in line with a structure–activity hypothesis based on observations with a variety of CsA derivatives, which highlighted the importance of the residues 11, 1, 2 and 3 for cyclophilin binding [29].

The extensive conformational differences between free and bound CsA [14,15,16,30] can be characterized as a transition from a structure with a maximal number of intramolecular hydrogen bonds to one that favours intermolecular hydrogen bonding with the environment. Thus, while in the crystal structure of CsA all four amide protons are involved in intramolecular hydrogen bonds, three of the four N-H groups in bound CsA point out into the solvent and the fourth, Abu-2, forms an intramolecular hydrogen bond with the hydroxyl group of MeBmt-1, which acts also as a hydrogen bond donor to the carbonyl oxygen of Asn-102 of cyclophilin. Since no hydrogen bond constraints were applied in the docking procedure, the formation of the intermolecular hydrogen bonds in the model was a result of the experimental intermolecular NOE constraints; the carbonyl oxygens of residues 1, 9, 10 and 11 and the hydroxyl group of MeBmt-1 in the binding face of CsA are all involved in intermolecular hydrogen bonds (Table II). The propensities of free and cyclophilin-bound CsA for hydrogen bond formation to a water environment were calculated [31], and the cyclophilin-bound conformation was found to have about 40% greater hydrogen bonding capacity than the hydrophobic form.

When comparing the binding modes of ac-Ala-Ala-Pro-Ala-amc in the crystal structure and of CsA in the presently described molecular model, one observes that the directions of the polypeptide chains in contact with cyclophilin are opposite to each other (Fig. 3B). Nonetheless, a best fit superposition of the cyclophilin backbone atoms in the two complexes shows that the pattern of hydrogen bonds and intermolecular contacts in the vicinity of the susceptible amide bond of the tetrapeptide substrate are conserved in the CsA complex. The only major difference is that although the amc group of ac-Ala-Ala-Pro-Ala-amc is well separated from Trp-121, this residue forms van der Waals and hydrogen bonding interactions with CsA. The hydrogen bond from Ala-2 NH to the carbonyl oxygen of Asn-102 observed in the crystal structure (which was also postu-

lated as part of a recognition motif [22]) is mimicked by the hydrogen bond between the MeBmt-1 side-chain hydroxyl group and the carbonyl oxygen of Asn-102 in the model of the CsA complex (Table II). Arg-55 of cyclophilin is also involved as a hydrogen bond donor in both structures, i.e. to MeLeu-10 C=O in CsA and to Pro-3 C=O in the linear peptide. As mentioned above, the isopropyl group of MeVal-11 in CsA and the proline ring of the linear tetrapeptide fit into the same hydrophobic binding pocket formed by residues Phe-60, Met-61, Phe-113 and Leu-122. The MeVal-11 C'-Cα bond of CsA and the *cis* amide bond between Ala-2 and Pro-3 of the tetrapeptide occupy corresponding positions in the two complexes. Overall, these observations show that the presently described model of the CsA-cyclophilin interaction does not support the previously postulated binding mode of CsA [6], according to which the hydroxyl group and the γ carbon of the MeBmt-1 side chain would mimic the positioning of the carbonyl oxygen and the amide nitrogen of an activated Xaa-Pro peptide bond.

Studies with mutant cyclophilins show that the high affinity binding in the CsA-cyclophilin system is mediated by interactions with residues that are not directly related to the site of enzymatic activity. In particular the Trp-121→Phe and Trp-121→Ala mutants of cyclophilin have been tested for CsA binding [32] and were found to be 75-fold and 200-fold less sensitive to CsA, respectively, which is consistent with our docking model. The concomitant drop in PPIase activity for these mutants is 2-fold and 13-fold, respectively. Cyclophilin from *E. coli* binds CsA weakly (IC_{50} = 3000 nM), but a Phe-121→Trp mutant of the *E. coli* protein was found to have 23-fold enhanced susceptibility to CsA inhibition [32]. A comparison of binding site residues in the *E. coli* protein with those determined from our structural studies suggests that the mutations Gln-72→Gly and Arg-101→Ala in the *E. coli* protein should further enhance CsA binding.

The work described here provides an example of how the complementary techniques of NMR spectroscopy, X-ray crystallography and computer-supported molecular modeling can be used in the drug design field. The results obtained provide insight into how CsA binds to cyclophilin and suggest novel experiments with modified CsA and mutant cyclophilins to modulate the strength of the interaction, and can thus support the design of new immunosuppressive drugs with modified pharmacological profiles.

REFERENCES

[1] Borel, J.F. (1989) Pharmacol. Rev. 41, 259–371.
[2] Schoenbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N. and Schmid F.X. (1991) J. Biol. Chem. 266, 3630–3635.
[3] Sigal, N.H., Dumont, F., Durette, P., Siekierka, J.J., Peterson, L., Rich, D.H., Dunlap, B.E., Staruch, M.J., Melino, M.R., Koprak, S., Williams, D., Witzel, B. and Pisano, J.M. (1991) J. Exp. Med. 173, 619–628.
[4] Liu, J., O'Farrer, J.D. Jr., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[5] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) Nature 341, 758–760.
[6] Schreiber, S.L. (1991) Science 251, 283–287.
[7] Michnick, S.W., Rosen, M.K., Wandless, T.J., Karplus, M. and Schreiber, S.L. (1991) Science 252, 836–839.
[8] Moore, J.M., Peattie, D.A., Fitzgibbon, M.J. and Thomson, J.A. (1991) Nature 351, 248–250.
[9] Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L. and Clardy, J. (1991) Science 252, 839–842.
[10] Kallen, J., Spitzfaden, C., Zurini, M.G.M., Wider, G., Widmer, H., Wüthrich, K. and Walkinshaw, M.D. (1991) Nature 353, 276–279.
[11] Ke, H., Zydowsky, L.D., Liu, J. and Walsh, C.T. (1991) Proc. Natl. Acad. Sci. USA 88, 9483–9487.
[12] Wüthrich, K., Spitzfaden, C., Memmert, K., Widmer, H. and Wider, G. (1991) FEBS Lett. 285, 237–247.
[13] Neri, P., Meadows, R., Gemmecker, G., Olejniczak, E., Nettesheim, D., Logan, T., Simmer, R., Hefrich, R., Holzman, T., Severin, J. and Fesik, S. (1991) FEBS Lett. 294, 81–88.
[14] Weber, C., Wider, G., von Freyberg, B., Traber, R., Braun, W., Widmer, H. and Wüthrich, K. (1991) Biochemistry 30, 6563–6574.
[15] Fesik, S.W., Gampe, R.T., Eaton, H.L., Gemmecker, G., Olejniczak, E.T., Neri, P., Egan, D.A., Edalji, R., Simmer, R., Helfrich, R., Hochlowski, J. and Jackson, M. (1991) Biochemistry, 30, 6574–6583.
[16] Loosli, H.R., Kessler, H., Oschkinat, H., Weber, H.P., Petcher, T.J. and Widmer, A. (1985) Helv. Chim. Acta 68, 682–704.
[17] Kessler, H., Köck, M., Wein, T. and Gehrke, M. (1990) Helv. Chim. Acta 73, 1818–1832.
[18] Zurini, M., Kallen, J., Mikol, V., Pfluegl, G., Jansonius, J.N. and Walkinshaw, M.D. (1990) FEBS Lett. 276, 63–66.
[19] Otting, G. and Wüthrich, K., (1989) J. Magn. Reson. 85, 586–594.
[20] Otting, G. and Wüthrich, K. (1990) Quart. Rev. Biophys. 23, 39–96.
[21] Wider, G., Weber, C., Traber, R., Widmer, H. and Wüthrich, K. (1990) J. Am. Chem. Soc. 112, 9015–9016.
[22] Kallen, J. and Walkinshaw, M.D. (1992) FEBS Lett. 300, 286–290.
[23] Schaumann, T., Braun, W. and Wüthrich, K. (1991) Biopolymers 29, 679–694.
[24] Ferrin, T.E., Conrad, C.H., Laurie, L.J. and Langridge, R. (1988) J. Mol. Graphics 6, 13–27.
[25] Insight / Discover Version 2.7, BIOSYM Technologies, 1991, San Diego, USA.
[26] K. Wüthrich, NMR of Proteins and Nucleic Acids, 1986, Wiley, New York.
[27] Braun, W. and Go-, N. (1985) J. Mol. Biol. 186, 611–626.
[28] Sanner, M. (1992) 'Modeling and Applications of Molecular Surfaces', Ph. D. thesis, Université de Haute Alsace, Mulhouse.
[29] Quesniaux, V.F.J., Schreier, M.H., Wenger, R.M., Hiestand, P.C., Harding, M.W. and Van Regenmortel, M.H.V. (1988) Transplantation 46, 23–27.
[30] Wüthrich, K., von Freyberg, B., Weber, C., Wider, G., Traber, R., Widmer, H. and Braun, W. (1991) Science 254, 953–954.
[31] Walkinshaw, M.D. and Floersheim, P. (1990) J. Mol. Struct. 237, 63–73.
[32] Liu, J., Chen, C.M. and Walsh, C.T. (1991) Biochemistry 30, 2306–2310.

APPENDIX

In previous papers we documented the NMR data used to obtain sequence-specific ^1H- and ^{15}N-resonance assignments for the polypeptide backbone in free [12] and CsA-bound [10] cyclophilin. Together with the NMR assignments for cyclophilin-bound CsA [14], these resonance assigments provided the basis for the spectral analysis of the intermolecular NOEs in the cyclophilin-CsA complex, and therefore the chemical shifts are presented in Table AI. In the table those residues are underlined for which the chemical shift differences between CsA-bound cyclophilin and free cyclophilin exceed any one of the following limits: 0.5 ppm for ^{15}N, 0.1 ppm for NH, 0.05 ppm for $C^{\alpha}H$.

For cyclophilin-bound CsA, chemical shifts of the backbone resonances were also reported by Neri et al. [13], who used slightly different measuring conditions, i.e. concentration of the complex 1.3 mM, solvent 50 mM phospate buffer containing 100 mM NaCl and 5 mM dithiothreitol, pH 6.5; *T*, 20°C. Furthermore, the ^{15}N chemical shifts were referenced to $H^{15}NO_3$, which causes a systematic difference of –0.5 ppm relative to the values in Table AI. Nonetheless, the two sets of chemical shifts are overall in good agreement: All ^{15}N shifts coincide within ± 1.0 ppm, except for Asp-27, Phe-36, Gly-50, Phe-112 and Phe-129, where the differences are in the range 1.0–1.5 ppm. All ^1H shift differences are smaller than 0.05 ppm, except that there is an outstandingly large difference of 0.63 ppm for $C^{\alpha}H$ of Glu-140, and that for the amide protons of Asp-27, Gly-65, Thr-68, His-70, Asn-71, Ser-77, Glu-81, Lys-82 and Val-128

Table AI

^1H and ^{15}N backbone chemical shifts of free and CsA-bound cyclophilin.*,**

Residue	δ(cyclophilin) ^{15}N	NH	(ppm) αH	δ(cyclophilin-CsA) ^{15}N	NH	(ppm) αH
Met-1						
Val-2			4.19			4.15
Asn-3	126.7	8.69	5.12	126.6	8.70	5.12
Pro-4			4.80			4.78
Thr-5	115.0	8.79	5.71	115.1	8.78	5.70
Val-6	120.3	8.76	5.32	120.7	8.74	5.30
Phe-7	119.1	8.98	5.89	119.3	8.98	5.88
Phe-8	116.9	9.57	5.30	117.0	9.57	5.28
Asp-9	124.1	9.29	5.52	124.1	9.28	5.52
Ile-10	124.3	9.06	5.18	124.3	9.06	5.15
Ala-11	132.4	9.63	5.15	132.4	9.63	5.14
Val-12	118.4	8.96	4.52	118.3	8.96	4.53
Asp-13	130.9	9.87	4.31	130.9	9.87	4.31
Gly-14	101.7	8.57	3.41,4.21	101.7	8.57	3.41,4.19
Glu-15	123.2	8.07	4.84	123.2	8.06	4.82
Pro-16			4.17			
Leu-17	125.9	9.21	4.70	126.0	9.20	4.68
Gly-18	102.3	7.72	3.95,4.26	102.3	7.26	3.94,4.25
Arg-19	121.2	8.37	5.62	121.2	8.37	5.61
Val-20	126.8	9.39	4.60	126.8	9.39	4.58
Ser-21	120.3	8.80	5.51	120.3	8.79	5.51
Phe-22	119.0	9.53	5.23	119.0	9.52	5.20
Glu-23	123.1	8.76	4.73	123.1	8.75	4.70
Leu-24	122.5	8.22	4.70	122.5	8.20	4.66
Phe-25	124.6	8.84	5.10	124.7	8.89	5.09
Ala-26	128.9	8.47	3.76	129.0	8.47	3.75
Asp-27	114.2	9.08	4.24	114.2	9.08	4.23
Lys-28	118.0	7.56	4.57	118.0	7.56	4.56
Val-29	114.5	8.39	4.44	114.7	8.38	4.42
Pro-30						
Lys-31	123.8	10.68	4.01	123.9	10.71	4.00
Thr-32	124.0	10.31	4.00	124.0	10.29	3.97
Ala-33	125.6	9.31	4.03	125.5	9.25	4.02
Glu-34	117.2	8.06	4.51	117.3	8.06	4.51
Asn-35	115.6	7.16	4.05	115.6	7.16	4.04
Phe-36	117.8	7.03	4.11	117.9	7.04	4.12
Arg-37	121.0	8.96	3.62	121.0	8.97	3.63
Ala-38	119.1	8.71	4.07	119.1	8.70	4.07
Leu-39	120.7	8.21	3.74	120.8	8.23	3.75
Ser-40	119.2	7.91	4.44	119.2	7.91	4.45

Table AI (continued)

^1H and ^{15}N backbone chemical shifts of free and CsA-bound cyclophilin.*,**

Residue	δ(cyclophilin) ^{15}N	NH	(ppm) αH	δ(cyclophilin-CsA) ^{15}N	NH	(ppm) αH
Thr-41	108.4	8.00	4.28	108.5	8.01	4.27
Gly-42	108.3	7.60	3.47,3.86	108.3	7.60	3.46,3.87
Glu-43	118.6	8.03	4.12	118.6	8.03	4.12
Lys-44	118.3	9.12	4.27	118.3	9.13	4.28
Gly-45	105.5	7.95	3.55,4.30	105.5	7.96	3.54,4.29
Phe-46	113.6	6.44	4.62	113.7	6.44	4.60
Gly-47	104.5	7.74	2.54,4.35	104.5	7.72	2.54,4.35
Tyr-48	113.9	6.93	4.20	113.9	6.89	4.19
Lys-49	124.9	8.50	3.59	124.9	8.50	3.57
Gly-50	117.8	9.50	3.68,4.39	117.9	9.52	3.68,4.39
Ser-51	116.3	8.41	4.64	116.6	8.43	4.62
Cys-52	115.1	9.96	5.89	115.0	9.90	5.90
Phe-53	123.0	8.72	4.85	123.6	8.70	4.85
His-54	120.1	7.64	4.74	119.7	7.54	4.70
Arg-55	123.0	7.06	5.10	124.2	6.92	5.07
Ile-56	126.4	9.18	4.60	127.1	9.12	4.58
Ile-57	127.9	8.75	5.20	127.2	8.50	5.25
Pro-58			4.32			4.37
Gly-59	114.1	9.75	3.79,4.04	113.9	9.78	3.83,4.05
Phe-60	119.1	8.19	5.09	119.1	8.11	[illegible]
Met-61	1[illegible]1	8.11	5.31	110.4	7.88	5.11
Cys-62	114.9	8.50	4.90	113.1	8.35	4.80
Gln-63	126.6	9.66	5.36	127.5	9.35	5.14
Gly-64	110.8	7.38		110.8	7.51	
Gly-65	105.9	9.35		105.0	9.05	
Asp-66	123.9	9.97	4.28	124.1	9.98	4.24
Phe-67	116.0	6.65	4.61	116.2	6.73	4.59
Thr-68	108.9	7.33	4.64	108.9	7.31	4.61
Arg-69	122.0	8.69	4.39	122.0	8.70	4.41
His-70	111.5	6.65		111.0	6.64	
Asn-71	112.3	7.53		112.3	7.50	
Gly-72	110.5	9.68	3.18,4.42	111.1	9.66	3.35,4.46
Thr-73	112.0	7.94	4.49	109.9	7.88	
Gly-74	113.9	8.72	2.55	113.9	8.78	3.46
Gly-75	108.9	8.12		108.8	8.07	
Lys-76	115.6	7.01	4.59	115.8	6.96	4.60
Ser-77	114.3	7.73	5.17	114.8	7.73	5.21
Ile-78	111.2	8.8[illegible]	4.17	111.1	8.88	4.17
Tyr-79	120.8	8.07	4.66	120.9	8.07	4.66
Gly-80	106.3	7.40	3.82,4.63	106.2	7.11	3.82,4.60

Table AI (continued)

^1H and ^{15}N backbone chemical shifts of free and CsA-bound cyclophilin.*,**

Residue	δ(cyclophilin) (ppm) ^{15}N	NH	αH	δ(cyclophilin-CsA) (ppm) ^{15}N	NH	αH
Glu-81				123.6	9.06	
Lys-82	111.8	7.81	5.56	111.8	7.82	5.56
Phe-83	116.4	9.17	4.92	116.3	9.18	4.92
Glu-84	119.5	9.29	3.77	119.3	9.29	3.76
Asp-85	118.8	8.62	4.22	118.8	8.64	4.23
Glu-86	131.7	9.48	3.79	131.7	9.54	3.79
Asn-87	106.8	7.07	4.13	106.9	7.07	4.10
Phe-88	112.9	8.35	5.93	112.8	8.33	5.94
Ile-89	119.9	8.32	3.64	119.9	8.32	3.64
Leu-90	117.1	8.76	4.46	117.1	7.78	4.45
Lys-91	118.9	8.08	4.63	118.9	8.07	4.60
His-92	122.5	10.72	4.35	122.3	10.57	4.31
Thr-93	110.3	7.29	4.01	110.4	7.28	3.99
Gly-94	107.2	7.50	3.60,4.36	107.3	7.56	3.60
Pro-95						
Gly-96	110.2	9.28	3.34,4.57	110.7	9.26	3.35,4.57
Ile-97	121.3	6.79	4.12	121.3	6.80	4.01
Leu-98	128.7	7.90	4.97	129.9	7.84	4.80
Ser-99	118.6	8.31	5.30	119.4	7.94	5.16
Met-100	123.0	8.57	5.28	123.2	8.32	5.50
Ala-101	126.0	8.03	4.28	126.9	8.25	4.70
Asn-102	113.2	8.14	4.58	117.6	7.69	
Ala-103	123.3	8.80	4.77	121.2	9.29	4.67
Gly-104	109.2	8.28	3.71,4.60	107.7	8.24	3.70,4.62
Pro-105			4.32			4.32
Asn-106	119.0	8.89	4.01	119.0	8.89	3.99
Thr-107	110.2	10.20	4.45	110.2	10.28	4.45
Asn-108	120.6	7.39	4.21	120.7	7.44	4.21
Gly-109	110.7	9.19	3.63,4.59	110.8	9.17	3.57,4.62
Ser-110	117.0	8.78	4.75	117.0	8.78	
Gln-111	124.3	8.41	5.16	124.7	8.44	
Phe-112	117.5	8.08	5.68	118.2	8.16	6.03
Phe-113	116.4	9.83	5.66	115.0	9.85	5.84
Ile-114	118.5	9.12	4.24	118.0	9.03	4.78
Cys-115	125.5	9.62	4.75	124.8	9.52	4.59
Thr-116	115.7	8.95	4.32	115.8	9.05	4.38
Ala-117	122.1	7.63	4.35	122.0	7.63	4.38
Lys-118	119.5	8.71	3.78	120.0	8.70	3.80
Thr-119	119.0	7.34	3.63	118.2	7.38	3.53
Glu-120	124.6	9.13	4.13	124.7	9.07	4.15
Trp-121	117.6	7.27	4.66	118.3	7.33	
Leu-122	119.8	7.02	4.26	120.7	7.07	4.30
Asp-123	122.1	7.63	5.17	122.3	7.61	5.16
Gly-124	111.1	9.56	2.98,3.98	111.1	9.59	2.98,3.98
Lys-125	115.3	7.72	4.12	114.7	7.60	4.07
His-126	119.6	7.59	4.75	120.3	7.46	4.80
Val-127	124.9	8.50	4.24	124.8	8.27	4.24
Val-128	133.0	9.51	4.15	132.9	9.39	3.98
Phe-129	117.8	8.14	5.28	117.8	8.13	5.19
Gly-130	110.7	7.32	2.98,3.15	110.8	7.38	3.10
Lys-131	115.2	8.38	5.22	115.0	8.33	5.16
Val-132	124.0	9.04	3.89	124.0	9.02	3.86
Lys-133	131.7	9.48	4.43	131.7	9.46	4.41
Glu-134	118.4	7.56	4.54	118.4	7.56	4.57
Gly-135	107.9	8.68	3.98,4.78	107.9	8.68	3.98
Met-136	122.5	8.87	4.44	122.6	8.86	4.44
Asn-137	114.4	8.93	4.46	114.4	8.93	4.42
Ile-138	124.2	7.66	3.74	124.2	7.66	3.73
Val-139	121.9	7.27	3.84	122.0	7.29	3.82
Glu-140	117.2	8.31	4.05	117.2	8.30	4.04
Ala-141	121.0	7.52	4.05	121.1	7.53	4.06
Met-142	117.6	8.29	3.98	117.6	8.32	3.97
Glu-143	116.3	7.86	3.72	116.4	7.90	3.73
Arg-144	114.5	7.04	3.89	114.9	7.05	3.92
Phe-145	115.3	7.63	4.80	115.2	7.63	
Gly-146	104.6	7.54	3.62,4.60	104.9	7.59	3.59,4.60
Ser-147	110.0	8.22	4.62	109.9	8.22	
Arg-148				120.3	8.93	4.16
Asn-149	111.4	7.88	4.85	111.4	7.87	4.86
Gly-150	110.2	8.06	3.93,4.10	110.7	8.07	3.94,4.15
Lys-151	119.9	7.55	4.42	119.8	7.54	4.41
Thr-152	116.7	8.86	5.53	116.8	8.86	5.56
Ser-153	117.0	9.44	4.45	117.3	9.48	4.42
Lys-154	119.2	7.55	4.55	119.2	7.55	4.52
Lys-155	121.8	8.82	4.31	121.8	8.83	4.28
Ile-156	134.2	9.61	5.09	134.1	9.60	5.07
Thr-157	117.2	9.26	5.28	117.2	9.24	5.28
Ile-158	121.7	8.60	4.23	121.8	8.59	4.20
Ala-159	132.5	8.90	4.13	132.5	8.90	4.11
Asp-160	111.5	8.08	4.90	111.5	8.09	4.87
Cys-161	116.1	8.62	4.54	116.1	8.62	4.54
Gly-162	104.0	6.86	3.55,3.90	104.0	6.86	3.52
Gln-163	121.0	9.09	5.06	121.0	9.08	5.04
Leu-164	125.9	8.63	4.61	125.9	8.62	4.60
Glu-165	125.9	8.15	4.17	125.9	8.14	4.15

*The concentration was 2 mM for the cyclophilin–CsA complex and 4 mM for the free protein; solvent: 90% H_2O, 10% D_2O, 10 mM $KOOCCD_3$, 10 mM KH_2PO_4, pH 6.0; T 25°C. The ^{15}N and ^1H chemical shifts were referenced to trimethylsilylpropionate, sodium salt (TSP).

**Those chemical shifts are underlined which differ between free and CsA-bound cyclophilin by ≥0.5 ppm for ^{15}N, ≥0.1 ppm for NH, and ≥0.05 ppm for $C^{\alpha}H$. The residue name is underlined whenever at least one of the shifts differences exceeds these limits.

and the C^{α} protons of Gly-50, Lys-76, Ile-89, Gly-94, Ala-103, Gly-104, Asn-106, Ile-114, Glu-140 and Gly-150 the chemical shift differences are in the range 0.05 to 0.15 ppm.