Modeling conformational changes in cyclosporin A

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Abstract

NMR and X-ray structures for the immunosuppressant cyclosporin A (CsA) reveal a remarkable difference between the unbound (free) conformation in organic solvents and the conformation bound to cyclophilin. We have performed computer simulations of the molecular dynamics of CsA under a variety of conditions and confirmed the stability of these two conformations at room temperature in water and in vacuum. However, when the free conformation was modeled in vacuum at 600 K, a transition pathway leading to the bound conformation was observed. This involved a change in the *cis* MeLeu-9 peptide bond to a *trans* conformation and the movement of the side chains forming the dominant hydrophobic cluster (residues MeBmt-1, MeLeu-4, MeLeu-6, and MeLeu-10) to the opposite side of the plane formed by the backbone atoms in the molecular ring. The final conformation had a backbone RMS deviation from the bound conformation of 0.53 Å and was as stable in dynamics simulations as the bound conformation. Our calculations allowed us to make a detailed analysis of a transition pathway between the free and the bound conformations of CsA and to identify two distinct regions of coordinated movement in CsA, both of which underwent transitions independently.

Keywords: cyclosporin A; dynamics; molecular modeling; molecular conformations; transitions

Cyclosporin A is an important therapeutic agent for the prevention of graft rejection in clinical organ transplantation. It is an 11-residue cyclic peptide of fungal origin (Dreyfuss et al., 1976) (Fig. 1) that contains two uncommon amino acids: (4R)-4-((E)-2butenyl)-4, N-dimethyl-L-threonine (Bmt) and L- α -aminobutyric acid (Abu), as well as several peptide bond *N*-methylated residues (residues 1, 3, 4, 6, 9, 10, and 11) (Wenger, 1983). CsA binds to a receptor called cyclophilin (CyP) and induces an interaction between the drug immunophilin complex CsA–CyP and the phosphatase calcineurin (Liu et al., 1992). The complex inhibits the signal transduction pathways that lead to T lymphocyte activation (Ryffel, 1989). The same immunosuppression occurs when the FK506–FKBP complex interacts with calcineurin (Liu et al., 1992), although both complexes are structurally unrelated.

The three-dimensional structure of CsA bound to CyP (Pfügl et al., 1993; Thériault et al., 1993) differs dramatically from that of free CsA in organic solvents (Loosli et al., 1985; Lautz et al., 1989, Kinemage 1), and the relationship between the two forms

has been described as “reminiscent of the inversion of a glove” (Wüthrich et al., 1991). The differences include a *cis*–*trans* transition of the peptide bond between MeLeu-9 and MeLeu-10 and the movement of the dominant hydrophobic cluster (formed by the side chains of residues MeBmt-1, MeLeu-4, MeLeu-6, and MeLeu-10) to the opposite side of the ring plane. The RMSD of the backbone atoms between the bound and free conformations of CsA is 2.43 Å. In order to activate the immunosuppressive effects of CsA, the molecule needs to be transported through the cell membrane and be placed within the cell cytoplasm. The ability of CsA to undergo a transformation may be critical for allowing it to pass through the nonpolar cell membrane. Once CsA partitions into the cell, binding of the “aqueous” form to CyP may shift the equilibrium back to the bound form. In the present paper, the conformation of CsA bound to CyP and the conformation in organic solvent will be referred to as the “bound” and the “free” conformations, respectively.

There have been a number of attempts to understand the forces that determine the two conformations, but the explanations are still the focus of much debate. It has been suggested that the free CsA conformation could be an artifact of the organic environment required to obtain the NMR structural data. The structure obtained using X-ray crystallography (Loosli et al., 1985; Kessler et al., 1990b) and neutron diffraction (Knott et al., 1990) required CsA in a crystallized form and it has been

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Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; RMSD, root mean square difference.

Fig. 1. Chemical structure of cyclosporin A (CsA) (Wenger et al., 1994) with the standard numbering of the amino acid residues. See text for description of the nonstandard residues MeBmt and Abu.

shown that the formation of crystal structures of small peptides or proteins can be different from the conformation in solution (Kessler et al., 1981). Experimental data obtained from NMR studies required CsA to be dissolved in nonpolar solvents such as chloroform (Loosli et al., 1985). Molecular dynamics studies of CsA in various solvents suggest that the hydrogen bonds will be formed with surrounding water molecules when in an aqueous solution and with each other in a nonpolar solution (Lautz et al., 1987). Allowing the amino acids to satisfy hydrogen bonds in this manner would result in a different preferred conformation (Kessler et al., 1990a). Studies of related proteins (Altschuh et al., 1992; Wenger et al., 1994) and studies of the binding velocity of CsA to CyP (Kofron et al., 1992) suggest the bound conformation may in fact be present in an aqueous solution in preference to the free conformation, which is found in organic solvents. However, despite the difference in data-gathering techniques of NMR and X-ray crystallography, the two methods do give a similar structure for the free conformation of CsA. Molecular dynamics studies of these two free conformation structures suggest that they converge to a single structure that is stable at room temperature in an aqueous solution (Lautz et al., 1987).

Another theory suggests that CyP is responsible for the transition of CsA to the bound conformation. It is possible that, as part of the process of binding to CyP, CsA is induced by CyP to undergo a conformational change from the free to the bound conformation (Jorgensen, 1991; Wüthrich et al., 1991; Altschuh et al., 1994). CyP is an enzyme with peptidyl-prolyl *cis-trans* isomerase activity (Fischer, 1994), although it appears that this

is unrelated to the change from *cis* to *trans* in the peptide bond between MeLeu-9 and MeLeu-10 (Thériault et al., 1993).

Finally, it is possible that both the free and bound conformations exist concurrently in an aqueous solution. A conformational change could be brought about either spontaneously or with the assistance of other atoms or ions contained in the solution (Carver et al., 1992; Köck et al., 1992). A number of studies suggest that, in addition to the two known conformations, CsA adopts at least five other "slowly interconverting" configurations in polar solvent environments (Kessler et al., 1985, 1990b; Loosli et al., 1985; Ko & Dalvit, 1992; Kofron et al., 1992; Thériault et al., 1993) and eight different conformations (including one with a *trans* MeLeu-9-MeLeu-10 peptide bond) have been observed in a 50/50 methanol/water mixture (Hsu et al., 1990). One study has indicated that there may be two conformations of CsA, both having a *trans* MeLeu-9-MeLeu-10 peptide bond, that bind to CyP (Kofron et al., 1992).

The conformational properties of CsA have been analyzed previously by unrestrained molecular dynamics simulations (Lautz et al., 1987, 1990; Gallion & Ringe, 1992), however, none of these reports detected major conformational transitions. Indeed, as pointed out by Wüthrich et al. (1991), when it became clear that the bound conformation was different from that of the already known free conformation, it was "instructive to note that molecular modeling ... gave no indication of ... [a] major conformation change" (Wüthrich et al., 1991).

A recent study (Köck et al., 1994) applied artificial constraints using a dynamic-forcing protocol to drive a free CsA conformation into a bound conformation. The constraints are first ap-

plied to residues MeBmt-1 and Val-5, driving the dihedrals to those found in the bound conformation, and, during the course of the trajectory, this force is applied incrementally to residues surrounding these two until, after 120 ps of simulation, the force is applied to the whole molecule. This results in a possible transition pathway. The resulting trajectory predicts a rotation of the MeBmt-1 side chain around the outside of the ring plane and the rotation of the Val-5 side chain through the center of the ring plane. The last step of the dynamic-forcing procedure is the *cis* to *trans* transition of the peptide bond between MeLeu-9 and MeLeu-10. The study, however, was unable to observe spontaneous crossings of the energy barriers that separate the free from the bound conformation.

Currently available computing resources limit molecular dynamic simulations to time periods in the pico- and nanosecond range. The transition time of CsA from a *cis* to a *trans* conformation is of the order of minutes (Kofron et al., 1992) and is likely to be missed by such short simulations. In order to overcome energy barriers more easily and thereby increase transition rates between different conformations, we decided to perform simulations at higher temperatures. This made it possible for us to observe conformational transitions within the time scale accessible to molecular dynamics. The empirical force field CHARMM22 (A.D. MacKerell & M. Karplus, in prep.), however, was derived for calculations at room temperature and, therefore, artificial, and even chemically forbidden, transition pathways may be observed at very high temperatures. To ensure that the observed transitions were also possible at room temperature, the lowest possible temperature where transitions occurred was sought by performing simulations at different temperatures.

Molecular dynamics simulations were also performed on two analogues of CsA. The first [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA is a water-soluble analogue of CsA with a known structure (Wenger et al., 1994). The free conformation of this molecule in polar solvent is nearly identical to the bound conformation of CsA. Simulations were performed to investigate the effects on the molecular dynamics properties caused by the chemical differences in this analogue. The second molecule (MeBm₂t)¹-CsA (Ke et al., 1994; Mikol et al., 1994) has a similar bound conformation and network of interactions to CyP as CsA. Its binding affinity for CyP is only 1% that of CsA, but it has an immunosuppressive effect that is greater than expected for its low binding affinity (Mikol et al., 1994). Because it has been suggested that this may be caused by a different equilibrium of free conformations in solution to CsA (Mikol et al., 1994), this molecule was also investigated. These analogues both raise the question of what effect modification of the side chains have on the conformational space occupied by the molecule. In order to try to answer this question, we applied the same modifications to both known conformations of CsA to study effects on the populations of backbone conformations observed during dynamics simulations.

Results

Stability at room temperature (300 K)

At 300 K, the dynamics trajectories for both the bound and free conformations of CsA in either a vacuum or an aqueous environment showed no major conformational change. Higher flexibility was observed in the MeLeu-9–MeLeu-10 peptide bond,

where the ω angle, which generally stayed within 30° of the mean value, would occasionally rotate up to a 60° deviation and then quickly rotate backward (see Fig. 2). During the 500-ps duration of these dynamics trajectories, the RMSD of the backbone atoms from the average position was less than 1 Å and, despite the flexibility of the peptide bond, there was no *cis* to *trans* or *trans* to *cis* transition (see Fig. 2). At room temperature, both

Fig. 2. Dial plots used in this article describe the change in backbone dihedral angles during the course of a molecular dynamics trajectory. The dihedral angle is plotted with a radius starting at zero at the beginning of the dynamics trajectory and increasing as the trajectory progresses until the final timestep is reached with a radius equal to that of the containing circle. During 50 ps of dynamics at 300 K, there is little change in any of the dihedral angles of the free conformation of CsA. The ω dihedral of MeLeu-9 shows increased flexibility.

the bound and free conformations of CsA were stable for 500 ps in both a vacuum and an aqueous environment. These results agree with previous molecular dynamics simulations for CsA (Lautz et al., 1987).

Conformational changes of CsA at 600 K

When the free CsA conformation was modeled in vacuum at 600 K, the *cis* peptide bond between residues MeLeu-9 and MeLeu-10 rotated to the *trans* conformation in 76 of 80 trajectories (Kinemage 2). The peptide bond rotated in a left-handed direction ($0 \rightarrow -90 \rightarrow 180$) (see the first 10 ps in Figs. 3, 4).

In 27 of the trajectories, a further rotational change of the MeLeu-9 peptide bond occurred. The MeLeu-9 peptide bond underwent a right-handed rotation, in some cases back to a *cis* conformation. In three of the simulations, the MeLeu-9 peptide bond underwent a complete right-handed 360° rotation, passing through the *cis* conformation and returning to the *trans* conformation. In 2 of 80 trajectories, the 360° rotations were followed a short time later (within 15 ps) by a right-handed rotation from -90° to $+90^\circ$ in the MeBmt-1 ψ angle (see Figs. 3, 4). In these two cases, the MeBmt-1 and MeLeu-10 side chains rotated slowly around the outside of the ring formed by the backbone atoms. The side chains continued rotating through the plane of the ring and adopted positions on the opposite side of the ring plane. This final orientation of the side chains of MeBmt-1 and MeLeu-10 and the backbone structure of residues 1–2, 9–11 resemble those of the bound conformation rather than the free conformation (see top right of Fig. 5).

When molecular dynamics calculations were continued beyond 50 ps for the structure containing the rotated MeBmt-1 and MeLeu-10 side chains, further conformational changes were observed. In 2 of 10 100-ps trajectories, the MeLeu-6 side chain rotated to the opposite side of the plane of the ring and the Val-5 side chain rotated through the center of the ring. This transformation was accompanied by changes in the MeLeu-4, MeLeu-6, and Ala-7 ψ angles (see Fig. 6 and Kinemage 2). Following a short time after these dihedral transitions (within 15 ps), the MeLeu-4 side chain rotated to the opposite side of the plane of the ring. This was correlated with a rotation of the MeLeu-4 ψ angle back to its original value and the Ala-7 ψ dihedral angle completing the transformation into the bound conformation (see Fig. 6). Following the previous conformational changes, the ψ angles of MeBmt-1 and MeLeu-10 also changed and this brought them more closely in line with the values found in the bound conformation of CsA (data not shown).

The resulting conformation contains a *trans* MeLeu-9 peptide bond and all four of the dominant hydrophobic side chains now occupy positions on the same side of the molecular ring plane as their counterparts in the bound conformation (see Fig. 7 and Kinemage 2). This transformed free CsA has all of the major characteristics of the bound CsA conformation and, when minimized and compared to the minimized bound conformation, the backbone atoms have an average RMSD of only 0.53 Å.

This final conformation was subject to a further 500-ps dynamics trajectory simulation at 300 K in both a vacuum and aqueous environment. During these trajectories, the RMSD of the backbone atoms from the average position was less than 1 Å and it was concluded that the new structure is essentially as stable at room temperature as the free or the bound conformation.

Fig. 3. During 50 ps of dynamics at 600 K, more flexibility in backbone dihedral angles of the free conformation of CsA can be observed. This plot shows the changes that occur as the side chains of residues MeBmt-1 and MeLeu-10 move to the opposite side of the molecular ring plane. The MeBmt-1 ψ and MeLeu-9 ω dihedrals are plotted in a dial plot of dihedral angles as the MeBmt-1 and MeLeu-10 residue side chains move to the opposite side of the ring plane.

During the molecular dynamics simulations, many conformations of CsA were observed where the backbone atoms had an average RMSD of greater than 2 Å from the two known conformations. The most notable of these had a change in the *cis* MeLeu-9 peptide bond to a *trans* conformation and a rotation of two of the four dominant hydrophobic cluster side chains (MeLeu-4 and MeLeu-6) to the opposite side of the molecule. In this half-inverted structure (bottom left-hand side of Fig. 5)

Fig. 4. Detail of the MeBmt-1 ψ (top) and MeLeu-9 ω (bottom) dihedrals (also displayed in Fig. 3) as the MeBmt-1 and MeLeu-10 residue side chains move to the opposite side of the ring plane. Dial plots for these angles are also shown as inserts.

the orientation of the MeLeu-4 and MeLeu-6 side chains and the backbone structure of residues 4–8 more closely resemble those of the bound conformation than that of the free conformation. When compared to the previously described half-inverted structure (see top right of Fig. 5), which was intermediate in the transition between the free and the bound conformations, the current structure has the opposite half of the molecule inverted. Both these structures where half of the molecule has inverted are stable at 600 K. When the dynamics simulation was continued for a further 500 ps at 300 K, in either vacuum or water, no substantial change in these conformations occurred.

As well as the two half inverted structures, other conformations were occasionally observed with only one of the dominant side chains rotated to the opposite side of the ring plane. These and other changes observed in the backbone dihedral angles gave rise to a large number of structures with minor conformational changes. Some of these structures also exhibited a degree of stability at 600 K and, when for two of them the calculations were continued for 500 ps at 300 K in both vacuum and water environments, no substantial change was observed.

A conformation containing a *cis* peptide bond between MeGly-3 and MeLeu-4 was observed in 4 of the 80 100-ps trajectories that started with the free conformation. However, by the time of the formation of this *cis* peptide bond, all four of these trajectories had lost the *cis* peptide bond between the MeLeu-9 and MeLeu-10. Only one of these four cases remained stable for the remaining 50 ps of the dynamics trajectory, the other three all immediately returned to a *trans* conformation.

Two regions of movement

Analysis of the covariance of the backbone atoms in the CsA trajectories reveals that there are two distinct regions of movement (see Fig. 8 and Kinemage 1). The movements of residues within each region are strongly coupled (i.e., they tend to move in the same direction at the same time). The two regions are: (1) MeBmt-1, Abu-2, MeLeu-9, MeLeu-10, MeVal-11; and (2) MeLeu-4, Val-5, MeLeu-6, Ala-7, D-Ala-8.

Region 1 coincides with the CyP binding loop of CsA and region 2, which faces into the solvent in the CyP–CsA complex, is involved with the interaction with calcineurin and coincides with the effector loop. It can also be seen (Fig. 8) that the movement of regions 1 and 2 exhibit a strong negative correlation (i.e., they tend to move in the opposite directions at the same time). When a video of the trajectory is inspected, the two regions are observed flexing apart then coming back together again. This correlation is strongest in the bound (Fig. 7, bottom), inverted (Fig. 7, center), and half-inverted conformations (Fig. 5). The covariance of atomic movements for the free conformation of CsA shows a similar behavior, although the magnitude of the movements is reduced.

Effect of temperature

Simulations of the free conformation at 300 K and 400 K showed increasing flexibility in the areas that produced the transitional changes at 600 K. At 400 K, in 3 of 10 50-ps simulations, there

Fig. 5. Stereo plots of CsA conformations in organic solvent (top left), bound to the receptor CyP (bottom right), and stable conformations observed during molecular simulations. The latter structures are conformations with either MeBmt-1 and MeLeu-10 side chains inverted (top right) or with side chains MeLeu-4 and MeLeu-6 inverted (bottom left) (see Fig. 2 for legend).

was a *cis* to *trans* change in the MeLeu-9–MeLeu-10 peptide bond, and, in one case, the MeLeu-4 and MeLeu-6 side chains rotated to the opposite side of the molecular ring plane (data not shown).

Effect of water at 600 K

Simulations for free CsA were also performed at 600 K in water, although the number of trajectories and their length was less than those performed in the vacuum simulations. However, in 6 of the 10 50-ps simulations, the *cis* peptide bond changed to a *trans* conformation and, although none of the side chains moved to the opposite side of the molecular plane, for short periods of time the MeLeu-4 and MeLeu-6 side chains rotated to a position that was parallel to the molecular ring plane.

The bound conformation

In simulations of the bound conformation at 300 K, the MeBmt-1 side chain unfolded slightly from the position on the back of the molecule that it occupies when bound to CyP. This orientation of the MeBmt-1 side chain is also found in the solution structure of the CsA–FAB complex, where “MeBmt-1 does not fold back onto the molecule as when CsA is bound to CyP” (Altschuh et al., 1992). When modeled at 600 K for 50 ps, in 8 of 10 trajectories the MeLeu-4 and MeLeu-6 side chains rotated to the opposite side of the molecular plane, however, in most simulations, these two side chains eventually returned to the original side. At no stage did the MeBmt-1 or MeLeu-10 residues change sides, nor was there a conformational transition (*trans* to *cis*) at the peptide bond between MeLeu-9 and MeLeu-10.

CsA analogues

The chemical differences between CsA and the two analogues [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA and (MeBmt₂)¹-CsA were ap-

plied to the two known structures of CsA. A comparison of the mean angular difference in backbone ϕ , ψ , and ω dihedral angles between the modified CsA analogues and wild-type CsA trajectories in the ensuing dynamics simulations found them to be similar to the average difference between two trajectories of wild-type CsA (see Table 1). The chemical modification of the side chains had little effect on the populations of backbone conformations observed during a dynamics simulation. The backbone conformations observed in the CsA analogues were found to be similar to those of the wild-type CsA upon which the analogue was based. These calculations show that, if the conformation of the analogue is near one of the two known backbone conformations of CsA, then chemical differences between the analogues and wild-type CsA have little effect on the backbone structure and dynamics of the molecules.

Although the simulations of CsA analogues were not as extensive as those performed on CsA alone, all of the simulations resulted in a distribution of structures that were similar to those of the corresponding CsA conformation on which they were based.

Discussion

The dynamics calculations we have performed at 600 K in vacuum have resulted in a trajectory that transformed a CsA molecule from the free conformation into a conformation essentially the same as the conformation bound to CyP. This gives support to the possibility that there is a defined transitional pathway between the two conformations. Simulations at lower temperature (400 K) also showed the movements that, at 600 K, result in conformational changes. This gives us some confidence that the changes we observed at 600 K would also occur at lower temperatures, but with less frequency and would be expected to occur over a longer time scale.

It is interesting to compare our results with those obtained by Köck et al. (1994), who employed a dynamic-forcing protocol.

Fig. 6. Changes observed in dihedral angles as the two remaining side chains in the dominant hydrophobic cluster, the MeLeu-4 and MeLeu-6 residue side chains, move to the opposite side of the ring plane. Shown are the MeLeu-4 (top), MeLeu-6 (center), and Ala-7 (bottom) ψ dihedral angles where movement was observed. Dial plots for these angles are also shown as inserts.

The two starting positions (MeBmt-1 and Val-5) they have chosen for applying the constraints correspond to the centers of the two regions of movement that we observe. Our unconstrained molecular dynamics simulations also show the MeBmt-1 side chain rotating around the outside of the molecular ring and of the Val-5 going through the center of the ring, although our results suggest that these movements may not happen simultaneously. In our simulations, the *cis-trans* transition of the peptide bond between MeLeu-9 and MeLeu-10 is found to be the first conformational change precipitating the rotation of the cyclophilin binding loop. This differs from the dynamic-forcing protocol, where this is the last step of the forced transition. In general, the dynamic-forcing protocol pathway seems to agree with our results apart from the timing of the changes in the *cis* peptide bond between MeLeu-9 and MeLeu-10.

A number of the simulated conformations for CsA were different from the two known conformations (two of these are

shown in Fig. 5). These conformations exhibited a similar degree of stability as the known conformations. The existence of these conformations in our calculations infers that the conformational space available to CsA in solution may not be restricted to the two conformations observed by X-ray crystallography and NMR, but may include a number of conformations. The structure of CsA in solution may better be described by a distribution of conformations between the accessible states. Individual CsA molecules would be constantly changing between members of this family of conformations. This agrees with experimental observations of CsA that indicate "the presence of several slowly interconverting conformations in aqueous solution" (Kessler et al., 1985, 1990a; Loosli et al., 1985; Ko et al., 1992; Kofron et al., 1992; Thériault et al., 1993). Changes in the environmental surrounding of CsA, such as placement in a nonpolar solvent, being bound to a receptor, or packed in a crystal structure, would understandably perturb the equilibrium of the conforma-

Fig. 7. Comparison of the backbone structure and the positions of the four dominant side chains in the free conformation of CsA (top); the conformation after 150 ps of modeling in vacuum at 600 K (middle); and the conformation of CsA that is bound to the receptor CyP (bottom). After 150 ps of modeling, the four dominant side chains have rotated to the opposite side of the molecular ring plane and the RMS difference of the backbone atoms with bound conformation is only 0.53 Å. The side chains shown are MeBmt 1 (dark green), MeLeu 4 (pink), MeLeu 6 (light green), and MeLeu-10 (pink). The backbone ring plane is shown as blue. MeLeu-9, the residue for which a *cis-trans* peptide bond transition is observed, is shown in yellow.

tional distribution, accentuating the occupancy of some conformations and even introducing new conformations.

The first part of the free conformation to undergo a conformational change was the MeLeu-9–MeLeu-10 peptide bond, which changed from a *cis* to a *trans* conformation. This change occurred in nearly all of the 600 K dynamic trajectories and was very rarely observed to change back to a *cis* conformation. The bound conformation, which already has a *trans* MeLeu-9–MeLeu-10 peptide bond, was never observed to change. The preference seems to be for a *trans* peptide bond in both the bound and free conformations. This leads to the possibility of conformations in solution that have a *trans* peptide bond between MeLeu-9 and MeLeu-10, as have been identified previously in NMR studies (Hsu et al., 1990; Kofron et al., 1992), but whose orientation of the side chains varies widely from that

of the bound CsA conformation and includes those that have a similar side-chain conformation to the free CsA conformation.

The major conformation population of CsA in organic solvents has a single *cis* peptide bond between MeLeu-9 and MeLeu-10. However, a minor conformational population (6%) has an additional *cis* peptide bond between the MeGly-3 and MeLeu-4 residues (Kessler et al., 1985, 1990b) and thiocyclosporin analogues also have a substantial (42%) conformational population in organic solvents containing these two *cis* peptide bonds (Seebach et al., 1991). In our simulations, a species containing both *cis* peptide bonds was never observed. However, *cis* peptide bonds were observed between the MeGly-3 and MeLeu-4 residues in a small number of the dynamics simulations (4/80). It is therefore likely that longer simulations would be able to detect a conformation of CsA with both *cis* peptide bonds. This result also shows that much more extended calculations would be needed to detect all possible conformations of CsA.

Aqueous solution simulations were not as extensive as those conducted in vacuum, due to the much increased requirement for computational resources. However, as with the vacuum simulations at 400 K, some of the precursors to the larger conformational changes, such as the *cis* to *trans* conformational change of the peptide bond between the MeLeu-9 and MeLeu-10 residues and the movement of MeLeu-4 and MeLeu-6 side chains, were observed. It is, therefore, likely that extended simulations studies in aqueous solution would result in a transition from the free to the bound conformation.

The behavior of CsA in inverting each half of the ring separately and the covariance analysis identifies two distinct regions of movement within the CsA molecule. These regions, one of which corresponds to the CyP binding loop and the other to the calcineurin effector loop, are hinged at the MeGly-3 and the MeLeu-9 residues. These hinges, which allow the molecule to flex back and forth (see Fig. 8), also separate the two functionally distinct domains of the molecule. The *cis* peptide bonds, when they are observed in CsA and its derivatives, also occur at these hinge regions. The separate movement of the two coordinated regions could result in large forces being applied to the hinge regions and this may localize enough energy to trigger a *cis-trans* transition.

It is noteworthy that the chemical differences between CsA and [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA occur near the flexible hinges of CsA. However, these modifications, when applied to both the free and the bound conformation of CsA, did not have any noticeable effect on the backbone conformations observed during dynamics simulations. These calculations support the hypothesis proposed by Wenger et al. (1994) that [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA is a realistic model for the structure of wild-type CsA, although it is not possible to quantify this from the results of these calculations.

Calculations performed for (MeBm₂t)¹-CsA in the free and the bound conformations also produced no noticeable differences in populations of backbone conformations. These calculations show no hint that the effect on binding affinity of (MeBm₂t)¹-CsA is due to a different equilibrium of free conformations in solution (Mikol et al., 1994). It is, however, not possible to quantify the occupancy of the bound conformer in comparison with other as-yet unexamined stable conformations that may be adopted by native CsA. More extensive calculations including CyP will have to be performed to understand the behavior of the CsA analogues.

Fig. 8. Covariance (see Equation 1) between backbone atoms in the bound conformation when modeled at 600 K. It shows clearly two distinct regions of movement. The insert schematic of CsA identifies the two regions of movement.

Molecular modeling is at an early stage of development and much work remains to be done in the area of parameter refinement and on the effectiveness of simulations in various environments. In spite of these deficiencies, molecular modeling is proving to be a useful additional technique to the modern molecular biochemist. In our case, it has indicated a potential pathway for the transition between the free and bound conformations of CsA, and, through identification of the conformational changes involved, it will help indicate ways to modify CsA which, for example, would lock the conformation into either the bound or free states.

Materials and methods

The coordinates of free CsA were obtained from the Cambridge Crystallographic databank (access code: DEKSAN) (Allen &

Kennard, 1993). They were determined from NMR data obtained from CsA in a CDCl_3 solution (Loosli et al., 1985). The bound conformation coordinates of CsA were obtained using NMR and X-ray data of the CsA-CyP complex (Pfögl et al., 1993).

Structure and force field for CsA

The obtained bound and free structures were used without change as the basis for molecular dynamics of CsA. Additional force-field parameters were required in order to describe the MeBmt and Abu residues in CsA. These consisted of the distribution of charges within the two residues and the force-field parameters required to describe the carbon-carbon double bond found in the MeBmt side chain. The distribution of charges was approximated by copying partial charges from those of atoms

Table 1. Mean angular difference in degrees of the backbone dihedral angles between trajectories^a

	CsA free 1	CsA free 2	CsA free 3	CsA bound	CsA(a) free	CsA(a) bound	CsA(b) free	CsA(b) bound
CsA free 1 ^b	49.9	48.1	49.6	67.8	53.3	66.8	53.7	67.4
CsA free 2 ^b	48.1	45.3	47.0	69.6	51.2	68.7	51.8	69.0
CsA free 3 ^b	49.6	47.1	47.8	68.5	52.6	67.6	51.5	68.0
CsA bound ^c	67.8	69.6	68.5	43.6	69.1	44.5	67.5	43.3
CsA(a) free ^d	53.3	52.0	52.6	69.1	54.6	68.5	55.9	68.9
CsA(a) bound ^e	66.8	68.7	67.6	44.5	68.5	44.3	66.6	44.2
CsA(b) free ^f	53.7	51.8	51.5	67.5	55.9	66.6	49.2	66.5
CsA(b) bound ^g	67.4	69.0	68.0	43.3	68.9	44.1	66.5	41.1

^a Each trajectory consists of 10 simulations each starting with the same initial conformation and undergoing 50 ps of molecular dynamics in vacuum at 600 K. The behavior of the analogues is similar to that of the native CsA conformation upon which they are based. See footnotes b–g for starting conformations.

^b CsA free, unbound (free) conformation of wild-type CsA in nonpolar solvents.

^c CsA bound, bound conformation of wild-type CsA.

^d CsA(a) free, (MeBm₂t)¹-CsA analogue with conformation based on the free wild-type CsA.

^e CsA(a) bound, (MeBm₂t)¹-CsA analogue with conformation based on the bound wild-type CsA.

^f CsA(b) free, [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA analogue with conformation based on the free wild-type CsA.

^g CsA(b) bound, [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA analogue with conformation based on the bound wild-type CsA.

in analogous chemical positions already parameterized in the force field, while ensuring that the total charge of the residues MeBmt and Abu were zero. Force-field parameters for the carbon double bond were obtained from the QUANTA package (Molecular Simulations Inc., Burlington, Massachusetts, USA). Where required, a methyl group was added to the standard residues without alteration of charges. The additional set of force field parameters (except for those obtained from QUANTA) used in these calculations are available upon request from the authors.

Structure and force field for CsA analogues

Structures for the two CsA analogues [D-MeSer³-D-Ser-(O-Gly⁸)]-CsA and (MeBm₂t)¹-CsA were obtained by patching the relevant residues in existing CsA structures. Patches were applied to both the bound and the free CsA conformation, resulting in two structures for each analogue. Force-field parameters as described for modeling CsA were also used to model the analogues and the distribution of charges in modified residues were also approximated from atoms' at analogous chemical positions.

Preparation for vacuum dynamics calculations:

To allow the initial CsA structures to relax in the vacuum environment, they were subjected to an energy minimization followed by 2.5 ps of molecular dynamics (at 300 K using an integration time step of 0.5 fs). These structures were then stored and used as a starting point for vacuum dynamics calculations.

Preparation for solvent dynamics calculations

A rectangular containment box was constructed around each of the CsA structures such that the sides of the box were at least 6 Å away from the nearest atom in the molecule. The box was filled with TIPS3P water molecules (Jorgensen et al., 1983) at 1 atmosphere pressure and a density of 0.0334 molecules/Å³

(Brünger, 1992). Water molecules within 2 Å radius of a heavy atom of CsA were then deleted. The CsA molecule was then fixed in position and, to allow the water molecules to equilibrate, a 5-ps dynamics run at 300 K using an integration time step of 2 fs was performed. Another minimization was then performed. A final 5-ps dynamics run at 300 K was then performed to allow the CsA to relax in the aqueous solution.

Dynamics calculations

The program XPLOR (Brünger, 1992) was employed in conjunction with the CHARMM22 force field (A.D. MacKerell & M. Karplus, in prep.) for all molecular dynamics simulations, energy minimization (Powell, 1977) and, in parts, for data analysis. During simulations, the SHAKE algorithm (Ryckaert et al., 1977) was used to keep all hydrogen bond lengths fixed. Water molecules were represented by the TIPS3P model of Jorgensen et al. (1983).

During all simulations, an integration time step of 1 fs was used (unless otherwise noted), a constant dielectric of 1.0 was employed, and the system was weakly coupled to a temperature bath (Berendsen et al., 1984; Brünger, 1992). In vacuum simulations, all nonbonded interactions were taken into account, whereas in solution they were truncated at 9 Å using a switching algorithm (Brünger, 1992).

The free and the bound conformations of CsA were subject to dynamics simulations in vacuum. Simulations were performed at room temperature and at a number of elevated temperatures. A large number of further simulations were performed at 600 K based on the free CsA conformation, where conformational changes resulting in half-inverted structures were observed. Some of the half-inverted structures were subject to further vacuum dynamics simulations at 600 K, and additional fully inverted conformations were observed in the trajectories. The resulting fully inverted conformation and two intermediate half-inverted conformations were then subject to final dynamics calculations at room temperature in water and in vacuum (see Table 2 for details).

Table 2. Different conditions for which molecular dynamics calculations were performed for CsA and CsA analogues

Initial conformation	Temperature (K)	Environment	Duration	Number of trajectories
CsA vacuum				
Bound CsA	300	Vacuum	50 ps	10
Free CsA	300	Vacuum	50 ps	10
	300	Vacuum	2.1 ns	1
	600	Vacuum	50 ps	80
	600	Vacuum	100 ps	80
	400	Vacuum	50 ps	10
Inverted free CsA structures (resulting from above dynamics)				
Free CsA with region 1 inverted	600	Vacuum	100 ps	20
	300	Vacuum	500 ps	1
	300	Aqueous	500 ps	1
Free CsA with region 2 inverted	600	Vacuum	100 ps	10
	300	Vacuum	500 ps	1
	300	Aqueous	500 ps	1
Free CsA completely inverted	300	Vacuum	500 ps	1
	300	Aqueous	500 ps	1
CsA in aqueous solution				
Free CsA	300	Aqueous	50 ps	10
	600	Aqueous	50 ps	20
Bound CsA	300	Aqueous	100 ps	1
	600	Aqueous	100 ps	1
CsA analogues				
Bound [D-MeSer ³ -D-Ser-(O-Gly ⁸)]-CsA	600	Vacuum	50 ps	10
	600	Aqueous	100 ps	1
Free [D-MeSer ³ -D-Ser-(O-Gly ⁸)]-CsA	600	Vacuum	50 ps	10
Bound (MeBm ₂ t) ¹ -CsA	600	Vacuum	50 ps	10
Free (MeBm ₂ t) ¹ -CsA	600	Vacuum	50 ps	20

Dynamic simulations were also performed in water starting with both the free and the bound conformations. Simulations were done at room temperature and at elevated temperatures. However, our calculations in water were not as extensive as the vacuum simulations (see Table 1 for details).

Dynamics simulations of the CsA analogues were performed at 600 K in vacuum. These were made for comparison with the wild-type simulations already performed (see Table 1 for details).

Analysis

For each of the dynamics run, all of the dihedral angles for the backbone residues were monitored. Periodically, for trajectories of interest or for comparison or additional analyses, the following attributes were monitored: total potential energy, the backbone RMSD from bound structure, and the backbone RMSD from the CsA free structure. Finally, the covariance of movement between all atoms was monitored.

The covariance between atom i and atom j was calculated as follows:

$$Cv_{ij} = \frac{C_{ij}}{\sqrt{C_{ii} \times C_{jj}}} \quad (1)$$

$$C_{ij} = \langle (R_i - \langle R_i \rangle) \times (R_j - \langle R_j \rangle) \rangle$$

R_i , positional vector of atom; $\langle R_i \rangle$, average position.

The covariance measures the correlated motion between pairs of atoms and has a range of -1 to $+1$. A value close to $+1$ indicates a strong in-phase correlation (i.e., the atoms tend to move in the same direction at the same time). A value close to -1 indicates a strong out-of-phase covariance (i.e., the atoms tend to move in opposite directions at the same time). Values near zero indicate that the movements are not correlated (Brünner, 1992).

The variabilities of structures both within a single dynamics trajectory as well as between two trajectories were determined by calculating and comparing the mean backbone dihedral angular difference between structures in the trajectories. In each dynamics trajectory, the backbone dihedral angles were stored every 0.25 ps. The backbone ϕ , ψ , and MeLeu-9 ω angles were compared for every pair of frames from two trajectories and the mean difference calculated. Trajectories for the purpose of these comparisons were composite trajectories containing 10 separate 50-ps dynamics trajectories, each starting from an initial structure, at 600 K in vacuum.

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