Lithium Chloride Perturbation of Cis-Trans Peptide Bond Equilibria: Effect on Conformational Equilibria in Cyclosporin A and on Time-Dependent Inhibition of Cyclophilin

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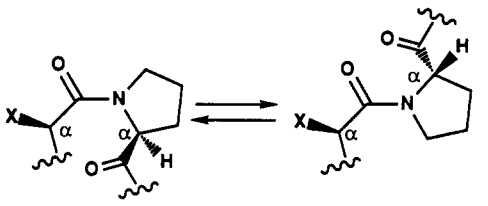
Abstract: The cyclic undecapeptide cyclosporin A (CsA) is a slow-binding inhibitor of the peptidylprolyl cis-trans isomerase (PPIase) cyclophilin. Both the initial inhibitory activity and the subsequent time-dependent inhibition are sensitive to the solvent system (DMSO, THF, LiCl-THF) in which CsA is dissolved prior to the assay. NMR experiments demonstrate that in tetrahydrofuran the MeLeu^9-MeLeu^{10} peptide bond has a cis conformation (Kessler, H.; et al. *Biochem. Pharmacol.* **1990**, *40*, 169-173). The cis conformer is inactive as a PPIase inhibitor. The same peptide bond adopts a trans conformation when lithium chloride is present as an additive in THF or when CsA is bound to cyclophilin (Fesik, S. W.; et al. *Science* **1990**, *250*, 1406-1409). The trans conformer is a tight-binding inhibitor of PPIase activity (K_i = 20 nM), and the inhibition increases over time (K_i = 7 nM after 30 min). Detailed kinetic analysis of this transition indicates the presence of at least two interconverting forms of unbound CsA, and a slow structural change in the enzyme-inhibitor complex. The kinetic and structural data taken together suggest that the sequence MeLeu^9-*trans*-MeLeu^{10}-MeVal^{11} is responsible for efficient binding in the active site of cyclophilin, while the corresponding cis conformer is not recognized at all. The interconversion between the two conformers is kinetically expressed in the time-dependent binding of the drug.

Introduction

We have recently reported that lithium chloride in anhydrous tetrahydrofuran (THF)[1] or trifluoroethanol (TFE) shifts the equilibrium between cis and trans Xxx-Pro peptide bonds in favor of the cis conformer (Scheme I).[2] In the case of the linear tetrapeptide Suc-Ala-Ala-Pro-Phe-pNa, the population of the cis Ala-Pro conformer increases from 10% to 40% when the conformer is dissolved in a solution of LiCl in THF (LiCl-THF), and to 70% when dissolved in a solution of LiCl in TFE. When the organic solvent-inorganic salt solutions of the substrate are added to the assay buffer, the increased amount of the cis Xxx-Pro conformer reverts to a population typical for aqueous environments (10-15% cis). This lithium chloride-induced conformational perturbation effect was used to develop a highly sensitive and versatile kinetic assay for monitoring the enzymatic activity of peptidylprolyl cis-trans isomerases (PPIases),[3] a new class of enzymes thought to play important roles in protein folding[4-10] and signal transduction.[11-13] The lithium chloride perturbation method allowed us to significantly improve the unfavorable signal-to-noise ratio of the established chymotrypsin-coupled spectroscopic PPIase assay.[3]

Several receptor proteins for the immunosuppressive drugs cyclosporin A (CsA, Figure 1), FK-506, and rapamycin[14-18] have peptidylprolyl cis-trans isomerase activity, and this enzymatic activity is inhibited by the drugs. In a previously reported kinetic study[2] we have shown that [Me^{5}Bth]^1-CsA, a moderately active CsA analogue (K_i = 45 nM), is a pure competitive inhibitor of cyclophilin, which indicates that the drug binds to the same kinetically significant molecular form of the enzyme as the substrate does. In the PPIase inhibition studies with [Me^{5}Bth]^1-CsA, the time course of substrate isomerization could be easily interpreted, because it conformed to a simple theoretical model of Michaelis-Menten kinetics accompanied by a parallel uncatalyzed process (the uncatalyzed cis-trans isomerization of Xxx-Pro bonds). When CsA itself was used as an inhibitor, however, the time course for the cis-trans isomerization of the substrate deviated from this simple theoretical model. Closer examination revealed that after the enzyme (cyclophilin) and the inhibitor (cyclosporin) were initially brought into contact, the apparent inhibition potency of the drug increased over time.[2] This process is usually referred to as "slow-binding inhibition".[19] The question arises as to what

Scheme I



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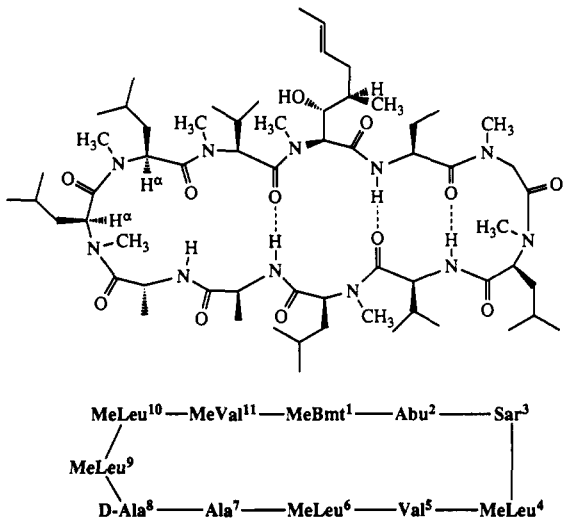

Figure 1. Schematic structure of the chloroform solution conformation of cyclosporin A (above) and the numbering system used for cyclosporin A analogues (below). The amide bond between MeLeu^9–MeLeu^{10} is cis in this figure.

is the structural correlate of the time-dependent binding.

It has been shown previously that when CsA is bound to cyclophilin, the MeLeu^9–MeLeu^{10} peptide bond has a trans conformation.[21] In contrast, the same peptide bond adopts a cis conformation in apolar solutions or in the crystals of the drug.[22,23] The typical activation energy for cis–trans isomerization in cyclic peptides is about 18–20 kcal/mol, which translates into transition times on the order of minutes. This value is comparable with the overall transition time which we observed in the time-dependent inhibition of cyclophilin by cyclosporin. We therefore hypothesized that the slow component in the binding of the drug was due to the slow interconversion between cis and trans conformers. In order to test the hypothesis, and characterize in detail the molecular processes involved in the time-dependent binding, we decided to take advantage of the lithium chloride-induced conformational changes in cyclosporin A. It was previously reported[24] that dissolving the drug in either THF or in a solution of LiCl–THF locks its conformation in two distinctly different states. Although the structure of CsA in THF has been characterized,[22,24] few details concerning the molecular structure of the conformer found in LiCl–THF were given.[24]

In this paper we show by isotope-edited NMR techniques that when CsA is dissolved in THF that contains 0.47 M LiCl, the tertiary amide bond between MeLeu^9 and MeLeu^{10} is trans rather than cis, as it is in solutions of THF alone. The cis 9,10 conformer of cyclosporin A has no inhibitory activity, whereas the trans 9,10 conformer is a tight-binding PPIase inhibitor. Further, the encounter complex between trans 9,10 CsA and cyclophilin rearranges over the course of ~30 min into a more tightly bound species. Analogous kinetic experiments in which cyclosporin was delivered to the assay buffer as a solution in DMSO rather than in THF indicate the presence of more than one inactive isomer. These solvent effects and lithium chloride-induced conformational perturbations are used here to characterize the conformation(s) preferred for efficient binding of cyclosporin to cyclophilin and to investigate kinetically the molecular processes responsible for the time-dependent binding of this important immunosuppressive drug to one of its receptors.

Experimental Section

Materials. Cyclosporin A was purified from a commercial preparation (Sandimmune) by silica gel flash chromatography and was 99% pure in HPLC and NMR analyses. The uniformly labeled ^{13}C analogue [U-^{13}C-*MeLeu*^{9,10}]CsA was prepared by modifications[25] of the method developed by Wenger.[26] The peptide inhibitors were dried in P_2O_5 in vacuo. The peptide substrate Suc-Ala-Ala-Pro-Phe-pNA was purchased from Sigma and used without further purification. THF was distilled over sodium–benzophenone; trifluoroethanol was distilled from sodium. LiCl was dried by heating to 250 °C in vacuo. All other materials were of reagent grade and used without further purification. Recombinant human cyclophilin (neutral form)[27] was a generous gift from Dr. Thomas Holzman (Abbott Laboratories).

PPIase Assays. A stock solution of cyclosporin A (1–2 mM) was prepared in dried THF and serially diluted to give a secondary stock solution 10 times more concentrated than the concentration desired in the enzyme assay (between 10 and 50 nM). The final 1:10 dilution was performed with DMSO, THF, or 0.47 M LiCl in THF (20 mg/mL). The substrate Suc-Ala-Ala-Pro-Phe-pNA was dissolved in 0.47 M LiCl in TFE. The population of the cis Ala-Pro conformer of the substrate was determined by the previously reported method[2] (typically 60–70% cis). A stock solution of cyclophilin (1.23 μM, 200 μL) was added to the thermally equilibrated buffer at 0 °C (20 mL; 50 mM Hepes–NaOH, 100 mM NaCl, pH 8.0 at 0 °C). The stock solution of the inhibitor (150–250 μL final concentrations 10–50 nM) was then added and the components were mixed. Aliquots (892 μL) of the enzyme–inhibitor mixture were withdrawn at various preincubation times and assayed for residual PPIase activity after addition of the substrate (8 μL) and chymotrypsin (100 μL) as described elsewhere.[2]

Determination of Initial Velocities. The initial velocities in PPIase assays were determined by nonlinear least-squares fit of time–absorbance data according to the method described earlier.[2] In the case of short enzyme–inhibitor preincubation times (between 1 and 5 min), a modified analysis was adopted that takes into account the slow binding inhibition. The system of first-order ordinary differential rate eqs 1–4 was integrated

$$-\frac{d[S]}{dt} = k_1[S] + \frac{k_{cat}[S]}{2([S] + K_m)}([E]_0 - [I]_0 - K_t' + \sqrt{([E]_0 - [I]_0 - K_t')^2 + 4[E]_0K_t'}) \quad (1)$$

$$K_t' = K_{if}' + (K_{in}' - K_{if}')e^{-k_s t} \quad (2)$$

$$K_{in}' = K_{in}(1 + [S]/K_m) \quad (3)$$

$$K_{if}' = K_{if}(1 + [S]/K_m) \quad (4)$$

numerically, by using the forward Euler method[28] with a step size of 0.5 s. In eqs 1–4, [S] is the concentration of the cis conformer of substrate at arbitrary time t, k_1 is the first-order rate constant for thermal cis-to-trans isomerization (0.002 s^{-1} at 0 °C), k_{cat} is the turnover number, K_m is the Michaelis constant, $[E]_0$ is the total concentration of the enzyme (11 nM), $[I]_0$ is the total concentration of the inhibitor (0–50 nM), and k_S is a first-order rate constant for the transition from the initial inhibited state, characterized by the apparent inhibition constant K_{in}, to the final inhibited state (inhibition constant K_{if}). The apparent time-dependent tight-binding inhibition constant K_t' was assumed as purely competitive; a series of preliminary kinetic analyses showed that the apparent time-dependent inhibition constant K_t' indeed decreased approximately exponentially with time. The system of ordinary differential eqs 1–4 represents an initial value problem; at time zero, the concentration of the cis conformer of the tetrapeptide substrate was equal to $[S]_0$. The concen-

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trations [S], calculated at mesh points identical with the experimental data, were converted to absorbance by using the molar absorption coefficient reported elsewhere.[2]

Models generated by numerical integration of eqs 1–4 were optimized to fit the experimental data by using the nonlinear least-squares method (Marquardt algorithm[29]), and the initial velocity was calculated by using eq 5, in which $[S]_0$ represents the initial concentration of the PPIase

$$v_0 = \frac{k_{cat}[S]_0}{2([S]_0 + K_m)}([E]_0 - [I]_0 - K_{in}'' + \sqrt{([E]_0 - [I]_0 - K_{in}'')^2 + 4[E]_0K_{in}''}) \quad (5)$$

$$K_{in}'' = K_{in}(1 + [S]_0/K_m) \quad (6)$$

substrate in the cis conformation. The apparent initial inhibition constant K_{in}'' in eq 5 is defined as in eq 6. The optimized parameters were K_m, k_{cat}, K_{in}, and K_{if}. The errors of estimation for the variable parameters were determined from the final curvature matrix as square roots of the corresponding variances.[30] The estimated variance of the initial velocity was calculated according to the error-propagation theory.[31] In addition to the variances $\sigma_{k_{cat}}^2$, $\sigma_{K_m}^2$, $\sigma_{K_{if}}^2$, and $\sigma_{K_{in}}^2$, all six corresponding non-diagonal elements of the covariance matrix were taken into account in the calculation of the error.

NMR Spectra. NMR data were acquired on a 9 mM sample of [U-^{13}C-*MeLeu*^{9,10}]-CsA in LiCl (470 mM)–THF-d_8 using a Bruker AMX500 (500 MHz) NMR spectrometer. The spectra were processed using a Bruker UXNMR software package on a Bruker X32.

The 2D HMQC–TOCSY experiment was acquired by using a standard HMQC pulse sequence[32] directly followed by an MLEV-17 TOCSY[33] mixing scheme (34 ms). The data were collected as 256 × 2048 complex points using a sweep width of 7462.7 and 10000 Hz in ω_1 (^{13}C) and ω_2 (^{1}H), respectively.

The HSQC spectrum[34] was acquired by using 128 × 2048 complex points and sweep widths of 7462.7 and 10000 Hz in ω_1 and ω_2, respectively.

The HMBC experiment was collected as previously described[35] using a delay of 20 ms to create antiphase ^{1}H–^{13}C magnetization. A total of 128 and 2048 complex points were acquired in t_1 and t_2, respectively. The sweep width was 10000 Hz in ω_2 (^{1}H) and 7462.7 Hz in ω_1 (^{13}C). The data set was processed in mixed mode[35] using an exponential line broadening (10 Hz) in ω_2 and shifted sine bell (45 Hz) in ω_1.

The isotope-edited 2D NOE spectrum[36–40] was collected by using a pulse sequence in which the signals corresponding to the protons attached to the ^{13}C-labeled nuclei of CsA were selectively observed in ω_1. The data were acquired as 160 × 2048 complex points using a sweep width of 7812.5 and 10000 Hz in ω_1 and ω_2, respectively. A total of 128 scans were acquired for each t_1.

Results

Structure and Kinetic Properties of CsA Dissolved in THF. Cyclosporin A in a THF solution (CsA–THF) adopts a single conformation in which the MeLeu^9–MeLeu^{10} peptide bond is cis.[22,24]

The cis 9,10 conformer is completely inactive as a PPIase inhibitor, as shown in Figure 2. The open square in Figure 2 indicates the velocity observed in the absence of inhibitors, and this velocity is equivalent to the value obtained by extrapolation of the CsA–THF series of experiments (filled circles) to zero preincubation time. When the inhibitor and the enzyme are allowed to equilibrate, the inhibition sets in and reaches its final value after about 25–30 min of preincubation. The values of velocities attained at equilibrium were measured at varied inhibitor concentrations and were also analyzed by nonlinear least-squares fit to the Morrison equation (5), in which the inhibition constant K_{in} (initial) was replaced by the inhibition constant K_{if} (final). The optimized value of the final inhibition constant K_{if} was 7 ± 2 nM.

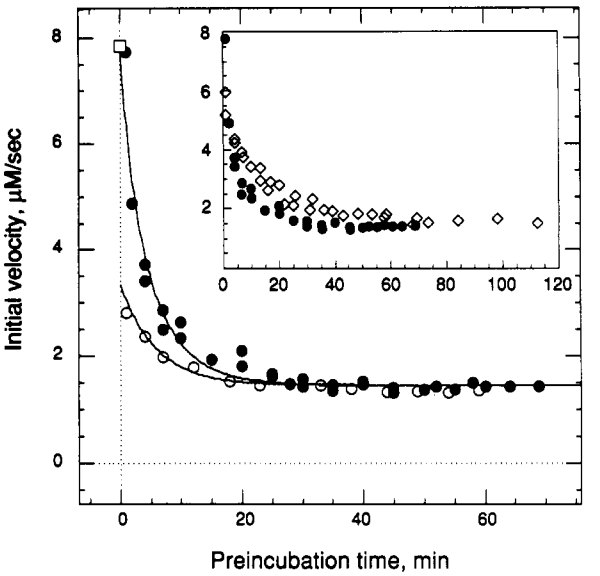

Figure 2. Initial velocities in the assays of recombinant human cyclophilin (11 nM) with Suc-Ala-Ala-Pro-Phe-pNa (105 μM, 60% cis - Ala-Pro-) as substrate,[2] in dependence on the enzyme–inhibitor preincubation time. The inhibitor (cyclosporin A, 33 nM) was dissolved in THF (filled circles) or 0.47 M LiCl–THF (open circles) prior to the beginning of the preincubation. The open square represents the initial velocity of an uninhibited assay under these conditions. The curves represent the results of nonlinear least-squares fit (Marquardt algorithm) of both data sets to the theoretical model represented by the system of ordinary differential eq 7–13. Inset: Results of similar experiments obtained by dissolving CsA in DMSO (diamonds) referenced against CsA in THF (filled circles).

Table I. ^{1}H and ^{13}C Chemical Shifts[a] of the MeLeu^9 and MeLeu^{10} Residues of [U-^{13}C-*MeLeu*^{9,10}]-CsA in LiCl (470 mM)–THF-d_8.

	MeLeu^9	MeLeu^{10}		MeLeu^9	MeLeu^{10}
C=O	171.1	174.5	H^γ	1.39	1.53
NCH_3	2.81	2.84	C^γ	25.9	26.3
NCH_3	30.6	31.8	H^δ	0.79	0.96
H^α	5.15	5.61	C^δ	23.6	24.0
C^α	54.7	50.9	$H^{\delta'}$	0.77	0.85
H^β	1.84/1.16	1.83/1.27	$C^{\delta'}$	23.2	21.6
C^β	39.2	39.0			

[a] Chemical shifts (ppm) are reported relative to TMS.

Structure and Kinetic Properties of CsA Dissolved in LiCl–THF. The structure of CsA in tetrahydrofuran containing 0.47 M lithium chloride was investigated by two-dimensional NMR techniques. The ^{1}H and ^{13}C NMR signals corresponding to the 9,10 MeLeu residues of [U-^{13}C-*MeLeu*^{9,10}]-CsA in LiCl–THF were assigned (Table I) from a 2D HMQC–TOCSY experiment and one-bond (HSQC) and multiple-bond (HMBC) ^{1}H–^{13}C correlation experiments. From an analysis of the 2D HMQC–TOCSY and HSQC spectra, the ^{1}H and ^{13}C signals corresponding to each of the MeLeu spin systems were identified. The NMe protons and carbonyl carbons connected to these spin systems were elucidated from intraresidue cross-peaks observed in the HMBC spectrum. To complete the assignments, the 9 and 10 carbonyl carbons were unambiguously distinguished by a 10NMe/9C′ HMBC cross-peak appearing as a doublet in the proton dimension due to the coupling to the ^{13}C spin of the NCH_3 group. The conformation of the MeLeu^9–MeLeu^{10} peptide bond was deduced from an isotope-edited 2D NOE spectrum of CsA dissolved in LiCl–THF. As shown in Figure 3, a NOE cross-peak was observed between the 9α- and 10NMe protons of CsA. However, no cross-peak was

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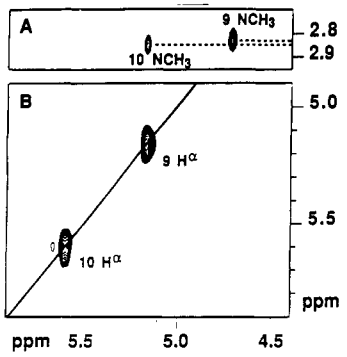


Figure 3. Portions of an isotope-edited 2D NOE spectrum of [U-^{13}C-*MeLeu*^{9,10}]-CsA in LiCl–THF. The lack of a NOE cross-peak between the 9α and 10α protons suggests that the 9,10 peptide bond is not in a cis conformation (B). Instead, when dissolved in LiCl–THF, CsA adopts a trans 9,10 peptide bond as evidenced by a 10NMe/9H$^{\alpha}$ NOE (A).

observed between the 9α and 10α protons. These NOE data indicate that CsA adopts a trans MeLeu^9–MeLeu^{10} peptide bond when dissolved in LiCl–THF. The 9,10 peptide bond of CsA has also been shown to adopt the trans conformation in the complex formed when CsA binds to cyclophilin.[21]

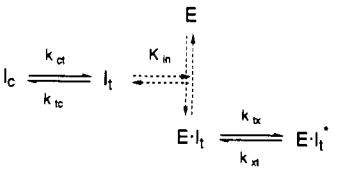
The kinetic properties of the trans 9,10 conformer of cyclosporin are indicated by the open circles in Figure 2. In contrast to CsA dissolved in THF alone, introducing the drug into the assay from a solution of LiCl–THF causes significant inhibition within the mixing time (10 s). The velocities extrapolated to zero preincubation time were measured at varied inhibitor concentrations (0–45 nM, 11 nM enzyme), and a nonlinear least-squares fit to the Morrison equation (5) yielded the initial inhibition constant for the cis isomer K_{in} equal to 20 ± 4 nM. As in the case of THF solutions, an equilibrium level of inhibition is reached after ~30 min. The inhibition constant for the corresponding final state, K_{if}, is identical to that obtained for CsA in THF. A complete kinetic analysis of the transition from the initial state to the final state is given in a separate section below.

Structure and Kinetic Properties of CsA Dissolved in DMSO. Cyclosporin A in DMSO adopts multiple conformational states; at least seven distinct conformations can be inferred from the ^{1}H NMR spectrum as judged by the multiplicity of NH and NMe proton peaks.[41] However, an unambiguous assignment of these conformers has not yet been achieved.

The kinetic behavior of CsA dissolved in DMSO (Figure 2, open diamonds in inset) shows similarities with the THF (filled circles) solution, but there are also two significant differences. First, as in the case with LiCl–THF, the reaction velocities extrapolated to zero preincubation time are lower than in the control experiment (with no inhibitor present). We did not attempt to determine the apparent initial inhibition constant K_{in} in this case, because it could not be ascribed to any one of the several cyclosporin conformations that exist in DMSO. Second, the overall transition times for achieving the equilibrium level of inhibition are longer than in the case of THF or LiCl–THF. These differences are readily apparent within first 60 min of preincubation time. In addition, we detected a "very slow binding" phase of the time-dependent inhibition (data not shown); when delivered into the assay in DMSO, cyclosporin consistently achieved a final (equilibrium) level of inhibition only after 12–16 h. Between 2 and 12 h of preincubation time, the residual PPIase activity decreased by approximately 5% and reached a value identical with that observed in the case of THF or LiCl–THF.

Estimated Activation Parameters for Time-Dependent Binding of Cyclosporin to Cyclophilin. We devised a theoretical model for the transition from the initial to the final inhibited state, which is represented by experimental measurements indicated by the filled circles (THF) and the open circles (LiCl–THF) in Figure 2. Fitting the observed initial velocities in dependence on the preincubation time to this model provided estimates for individual activation energies involved in the time-dependent binding. The most economical theoretical model is represented in Scheme II. It accounts for the following two observations: first, the cis 9,10 conformer of cyclosporin (I_c) does not bind, since CsA in THF has no inhibitory activity; second, the trans 9,10 conformer (I_t) found in LiCl–THF has some initial affinity, immediately after being bought into contact with the enzyme, but the binding increases over time, most likely because the initial enzyme–inhibitor complex ($E{\cdot}I_t$) is converted into a more tightly bound species ($E{\cdot}I_t^*$). The bimolecular step in Scheme II (binding constant K_{in}) is assumed to be very rapid in comparison with all other monomolecular steps involved (rate constants k_{tc}, k_{ct}, k_{tx}, and k_{xt}). This assumption is justified, because the diffusion-controlled bimolecular association rate constant k_{bi} for cyclosporin and cyclophilin is likely to be approximately 10^8 M^{-1} s^{-1}, so that at 10 nM enzyme the pseudo-monomolecular rate constant $k_{bi}[E]_0$ is 1 s^{-1}. The dissociation rate constant for the initial enzyme–inhibitor complex, $k_{bi}K_{in}$, can be estimated at ~2 s^{-1} because the initial inhibition constant determined experimentally is 20 nM (see above). On the other hand, the time-dependent binding occurs with an apparent half-time of ~15 min, which corresponds to an apparent first-order rate constant of 10^{-3} s. Thus the monomolecular processes involved in the time-dependent binding are most likely ~1000 times slower than the rate of association and dissociation of the enzyme–inhibitor complex. Under the assumption of rapid equilibrium, the multiphasic binding of cyclosporin A to cyclophilin can be described in a "hybrid" numerical integration scheme. The differential eqs 7–10 are integrated by using the forward Euler method. However, within each integration step, the rapid bimolecular equilibrium is enforced according to eqs 11–13, where $[E]_0$ and $[I]_0$ are the total concentrations of the enzyme ($[E]_0 = [E] + [E{\cdot}I_t]$) and the inhibitor ($[I_t]_0 = [I_t] + [E{\cdot}I_t]$) that participate in the equilibrium.

Scheme II



$$d[E{\cdot}I_t]/dt = k_{xt}[E{\cdot}I_t^*] - k_{tx}[E{\cdot}I_t] \quad (7)$$

$$d[E{\cdot}I_t^*]/dt = k_{tx}[E{\cdot}I_t] - k_{xt}[E{\cdot}I_t^*] \quad (8)$$

$$d[I_t]/dt = k_{ct}[I_c] - k_{tc}[I_t] \quad (9)$$

$$d[I_c]/dt = k_{tc}[I_t] = k_{ct}[I_c] \quad (10)$$

$$[E] = ([E]_0 - [I_t]_0 - K_{in}' + \sqrt{([E]_0 - [I_t]_0 - K_{in}')^2 + 4[E]_0K_{in}'})/2 \quad (11)$$

$$[I_t] = ([I_t]_0 - [E]_0 - K_{in}' + \sqrt{([I_t]_0 - [E]_0 - K_{in}')^2 + 4[I_t]_0K_{in}'})/2 \quad (12)$$

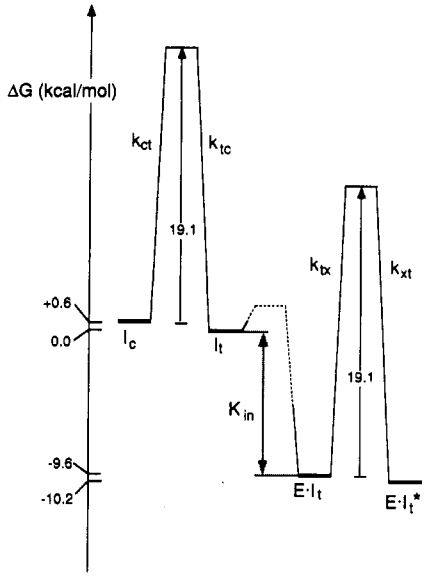
$$[E{\cdot}I_t] = [E][I_t]/K_{in} \quad (13)$$

Initial velocities measured at different preincubation times are proportional to the concentration of the free enzyme [E], which is calculated in the hybrid numerical integration scheme. Nonlinear least-squares fit of the initial velocities vs preincubation time to the model represented by the system of eqs 7–13 allows an approximate estimation of the rate constants k_{ct}, k_{tc}, k_{tx}, and k_{xt} and thus the estimation of free energy barriers for the interconversion of various forms of cyclosporin A, both free in solution and bound to the enzyme. In the nonlinear fit, the initial inhibition constant K_{in} was assumed constant and equal to 20 nM. One of the kinetic constants k_{xt}, k_{tx} could be expressed in terms of other constant in this pair, and in terms of the final inhibition constant K_{if} (held at a constant value of 7 nM) according to eq 14; the

$$K_{if} = K_{in}k_{tx}/(k_{tx} + k_{xt}) \quad (14)$$

(41) Kessler, H.; Oschkinat, H.; Loosli, H.-R. *Two-Dimensional NMR Spectroscopy*; Verlag Chemie: Weinheim, 1987; p 259.

Scheme III



optimized parameters of the model were the three rate constants k_{ct}, k_{tc}, and either k_{xt} or k_{tx}. The variances of optimized parameters were calculated from the final curvature matrix within Marquardt's nonlinear least-squares algorithm. The optimized values of rate constants were $k_{ct} = (3.4 \pm 0.4) \times 10^{-3}$ s^{-1}, $k_{tc} = (1.2 \pm 0.2) \times 10^{-3}$ s^{-1}, $k_{tx} = (3.4 \pm 0.7) \times 10^{-3}$ s^{-1}, and $k_{xt} = 1.2 \times 10^{-3}$ s^{-1}. Both data sets shown in Figure 2 were fitted simultaneously, and the fitted curves are indicated there.

The activation energies were calculated from the monomolecular rate constants according to transition-state theory, and the results are shown in Scheme III. The activation barrier for the interconversion between the 9,10 cis and trans conformers of cyclosporin A is approximately 19 kcal/mol, in agreement with the value usually observed for cyclic peptides. The activation barrier for the formation of the tightly bound enzyme inhibitor complex from the species formed initially is also approximately 19 kcal/mol.

Discussion

The slow onset of PPIase inhibition by cyclosporin A suggests that the drug is slowly equilibrating between more active and less active conformers prior to binding to the enzyme. In addition, it is very likely that the enzyme–inhibitor complex undergoes similar conformational transitions. Our earlier observations of the time-dependent inhibition of cyclophilin[2] involved DMSO as solvent for CsA, but those early results were impossible to interpret quantitatively because CsA in DMSO and in protic solvents exists as an ensemble of many conformers. The same applies for several CsA analogues. We have synthesized a water-soluble amine derivative of CsA, [D-diaminobutyric acid]^8-CsA ([Dab]^8-CsA), and shown that it adopts several slowly interconverting conformations in aqueous solution (data not shown).

In order to simplify the kinetic analysis, we considered ways to restrict the CsA conformation prior to adding the inhibitor to the aqueous assay buffer. Under favorable conditions, it is possible to separate conformers of biologically active molecules and measure their activities. This was first achieved with conformations of the cyclic tetrapeptide D-MeAla-tentoxin, which could be separated by TLC at low temperature.[42] Separation and subsequent bioassay of D-MeAla-tentoxin conformers as inhibitors of chloroplast coupling factor I[43] could be achieved because the conformational barrier was relatively high (22 kcal/mol).[42] Similarly, separation of cis and trans Ala-Pro conformers of the ACE inhibitor ramiprilat by HPLC has also been reported.[44] However, our attempts to isolate different conformations of CsA that exist in methanol by low-temperature TLC were unsuccessful. Although multiple conformations could be clearly detected, these interconverted even at low temperature and isolation was not practical.[45] Thus, we attempted to sequester one or more conformations of CsA by solvent-induced effects. Kessler and coworkers reported that, in the presence of LiCl–THF, cyclosporin A adopts one major conformation that is different from the conformations that exist in chloroform and in tetrahydrofuran.[24] By carrying out isotope-edited NMR studies on the isotopically labeled [U-^{13}C]MeLeu^9-[U-^{13}C]MeLeu^{10} cyclosporin A,[21] we established that in the lithium chloride-complexed conformer, now fully characterized in the accompanying paper by Köck et al.,[46] the 9,10 amide bond is trans. *Thus, the absence or presence of LiCl in THF sets the 9,10 peptide bond conformation in CsA cis or trans, respectively, at the time of addition to the aqueous buffer.*

The use of LiCl–THF and THF alone to sequester different conformations of CsA provides a method to obtain some of the individual kinetic constants that characterize the time-dependent inhibition of cyclophilin. Our results show that the cis MeLeu^9-MeLeu^{10} conformer, generated by dissolving the drug in THF, has no inhibitory activity. On the other hand, the trans MeLeu^9-MeLeu^{10} conformer found in LiCl–THF is a tight-binding PPIase inhibitor (K_{in} = 20 nM), and furthermore, the apparent inhibition constant decreases over time (K_{if} = 7 nM after 30 min). The activation barrier for achieving the final inhibited state is approximately 19 kcal/mol. It is interesting that this energy barrier is roughly equivalent to the activation barrier for cis–trans isomerization of a peptide bond. It should be noted that there is an alternative, although less likely, explanation for the apparent increase over time in the inhibitory activity of the trans 9,10 conformer found in LiCl–THF. It is possible that the gradual change in the apparent inhibition constant is due to a slow formation of a secondary, more potent form of the free inhibitor, rather than of a rearranged enzyme–inhibitor complex. At this stage we are unable to distinguish between the two possibilities due to the complexity of the numerical analysis. Also, the simplified model does not address the possible existence of more than one inactive or partially active conformers besides I_c, even though such conformers are likely to exist in aqueous solutions.

When CsA is dissolved in methanol, ethanol, or dimethyl sulfoxide[47] before being added to cyclophilin in the aqueous buffer, the time-dependent inhibition involves a markedly slow component (Figure 2, inset). These solvents are commonly used to dissolve the drug and its analogues in biological assays (immunosuppression) and biochemical assays (PPIase inhibition). If not properly taken into account, the unusually slow onset of inhibition could cause an artifactually lowered biological activity. Perhaps at least some of the variance among PPIase inhibition constants for cyclosporin analogues reported in the literature could be attributed to an insufficient preincubation time. In some extreme cases (e.g., [Dab(Boc)]^8-CsA delivered in DMSO),[2] the equilibrium level on PPIase inhibition was attained only after 16 h of preincubation; the decrease in residual PPIase activity between 2 and 16 h of preincubation amounted to 15%, as compared to 5% in the case of THF. Obviously, an insufficiently long preincubation of this inhibitor with the enzyme would yield an underestimated K_i. Similarly, since many of the cell changes initiated during calcium-mediated signal transduction occur within 60–120 min following stimulation,[48] some CsA analogues may not have reached equilibrium in binding to cyclophilin or other receptors during that time period.

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(45) Kishore, V. unpublished data.
(46) Köck, M.; Kessler, H.; Seebach, D.; Thaler, A. *J. Am. Chem. Soc.*, following paper in this issue.
(47) From experiments performed with a limited set of CsA analogues, the time course for inhibition with the compounds dissolved in DMSO, ethanol, or methanol is identical (data not shown).
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Our kinetic results establish that the trans MeLeu^9-MeLeu^{10} conformer of CsA, which exists as the sole structural form in LiCl–THf, has a fairly high affinity toward cyclophilin (K_{in} = 20 nM). In the cyclosporin–cyclophilin complex, the conformation of MeLeu^9-MeLeu^{10} is also trans.[21] It is interesting to compare the conformations of the lithium-complexed species with the conformation of receptor-bound cyclosporin in more detail. Köck et al. point out in the accompanying paper in this issue[46] that the side-chain torsion angles for the residues -MeLeu^9-MeLeu^{10}-MeVal^{11}- are remarkably similar for the lithium-complexed and for the receptor-bound conformers, but that the side-chain torsion angles for residues MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^6- are very different. Consequently, the cyclosporin sequence -MeLeu^9-MeLeu^{10}-MeVal^{11}- in the proper conformation induced by LiCl–THF is responsible for efficient initial binding to the receptor. The time-dependent phenomena observed in our inhibition studies with LiCl-complexed cyclosporin A are likely to arise from conformational changes in cyclosporin residues MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^5-.

Some of the very slow processes observed in the inhibition studies are difficult if not impossible to interpret in structural terms, for example the 16-h equilibration of [(Boc)Dab]^8-CsA delivered into the assay in DMSO. Detailed kinetic investigations are hindered by low aqueous solubility of these very hydrophobic molecules. In fact, the extreme hydrophobicity of many CsA analogues may be at the root of their anomalously slow conformational transitions in water. Strong hydrophobic interactions between cyclosporin side chains may firmly lock the molecule in an ensemble of conformations, some of which may have low binding affinity. For example, the orientation of the MeBmt^1 side chain in the lithium-complexed conformer is very different from the cyclophilin-bound conformation. Consequently, this very hydrophobic side chain may be trapped in a nonoptimal orientation when cyclosporin, originally dissolved in LiCl–THF, is introduced into the aqueous buffer. Similar considerations apply for other hydrophobic residues (Abu^2, MeLeu^4, and Val^5) whose conformations are different in LiCl–THF and in the enzyme-bound state.

We have previously shown by enzyme kinetic methods that the inhibition of cyclophilin by [Me^5-MeBth]^1-CsA conforms to a purely competitive kinetic mechanism;[2] recent biophysical data have provided the opportunity to evaluate our conclusion that cyclosporin and its analogues probably bind in the PPIase active site. In the X-ray crystal structure of human cyclophilin complexed with the tetrapeptide substrate Ac-Ala-Ala-Pro-Ala-amidomethylcoumarin, the tetrapeptide is bound within a long, deep grove close to Trp^{121} located at its base.[49] On the basis of chemical shift differences between uncomplexed cyclophilin and cyclophilin bound to a water-soluble CsA derivative, it was postulated that CsA also binds in this region. Changes in cyclophilin chemical shifts, however, may arise either from a direct interaction between CsA and cyclophilin or from a conformational change in the protein region distant from the CsA binding site. The isotope-edited NOE data of Fesik and co-workers[21,50] established that MeLeu^9 in cyclophilin-bound cyclosporin is also very close to the Trp^{121}. More recently, additional CsA–cyclophilin NOEs that were previously observed in ^{13}C-resolved 3D HMQC-NOESY spectra of uniformly ^{13}C-labeled CsA bound to cyclophilin[50] were assigned and were used to orient CsA in the cyclophilin binding site.[51] Thus the predictions based on the competitive inhibition pattern for [Me^5-MeBth]^1-CsA have been confirmed by structural methods: both the tetrapeptide substrate and cyclosporin A as inhibitor bind in the PPIase active site. It is interesting to note some subtle differences in the relative orientations of the substrate and the inhibitor. In the complex with the tetrapeptide substrate, Trp^{121} in cyclophilin lies close to the *C-terminal* coumarin group, but in the complex with cyclosporin A, the same Trp^{121} is close to the *N-terminal* MeLeu^9 of the active site binding region (-MeLeu^9-MeLeu^{10}-MeVal^{11}-MeBmt^1-Abu^2-Sar^3-MeLeu^4-). That is, the substrate and the inhibitor are oriented in opposite directions relative to each other.

The existence of multiple conformations of cyclosporin reported herein can influence the biological properties of the drug in subtle ways. On one hand, it suppresses the apparent affinity for the target enzyme, cyclophilin, and on the other hand, it increases the likelihood of interactions with other receptor systems. In the present study, we have only examined the effect of isolated conformations of CsA on PPIase inhibition. The conformational effects that promote binding to proteins other than cyclophilin[52-58] have not yet been determined, and it is conceivable that conformations that bind weakly to PPIases could be important in these cases. The CsA–cyclophilin system illustrates a new approach to explore conformational interconversions of peptides, by using the LiCl–THF perturbation method to selectively alter both biological and physical properties of peptide ligands. The ability to control the conformation of peptides by using the effect of solvents and solvent–salt solutions represents a novel, facile methodology that should find wide applicability.

Acknowledgment. This work was supported by grants from the National Institutes of Health AR32007 and AI24650.

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