Hydration Changes Implicated in the Remarkable Temperature-Dependent Membrane Permeation of Cyclosporin A

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ABSTRACT: Cyclosporin A is a cyclic peptide believed to exist as multiple conformers in aqueous solution. Two major conformations, distinguished by a single cis−trans isomerization and the presence of four either intramolecular or intermolecular hydrogen bonds, have been confirmed depending on whether CsA is characterized in organic solvents or bound in aqueous complex with cyclophilin. The relationship between CsA conformation and its ability to penetrate biological membranes is currently unknown. Using Caco-2 cell monolayers, we documented a remarkable increase (more than 2 orders of magnitude) in the membrane permeation of the peptide as temperature was increased from 5 to 37 °C. The solubility of CsA was 72 μM at 5 °C, but decreased by more than an order of magnitude at 37 °C. Moreover, CsA partitioned into non-hydrogen bond donating solvents linearly as a function of increasing temperature, suggestive of a significant conformational change. However, while NMR spectra of CsA confirmed the previously predicted presence of multiple conformers in aqueous solution, the equilibrium between the two major species was not affected by changes in temperature. These NMR data indicated that the observed temperature-dependent changes in the membrane permeability of CsA do not originate from changes in the peptide backbone conformation. Sedimentation equilibrium analysis revealed that CsA behaves in a highly nonideal manner over the temperature range tested. We interpret this behavior as a change in the hydration state with a smaller (or weaker) hydration shell surrounding the peptide at higher temperatures. Such a change would result in lower peptide desolvation energy, thereby promoting partitioning into cellular membranes. We contend that changes in membrane penetration result from alterations in the hydration state of CsA and are not related to the interconversion of the defined conformations.

Cyclosporin A (CsA)[1] is an unusual peptide drug in that it is administered orally. Currently the agent of choice for immunosuppression therapy, large interpatient differences in bioavailability are encountered with its use (*1*−*3*). Several factors have been put forth to account for these bioavailability differences including limited drug solubility, poor intestinal permeability, and first pass metabolism during transport through the intestinal mucosa and liver (*2*, *4*−*9*). To explore mechanisms related to the oral bioavailability of CsA, we have used Caco-2 cell monolayers (*10*, *11*) as an in vitro membrane model of the human intestinal epithelium. Our work demonstrated that CsA crosses Caco-2 monolayers by passive diffusion, partially opposed by a polarized efflux system located at the apical membrane (*12*). Participation of this MDR-related P-glycoprotein (P-gp) pump is probably another key physiological determinant of the oral bioavailability of CsA.

The CsA molecule is a cyclic undecapeptide (Figure 1) of fungal origin (*13*, *14*). This interesting structure contains two unusual amino acids: (4*R*)-4-((*E*)-2-butenyl)-4,*N*-dimethyl-L-threonine (MeBmt) and L-α-aminobutyric acid (Abu). Additionally, seven of the 11 amide bonds are *N*-methylated, contributing to the extreme hydrophobicity associated with the peptide (*15*). Two major conformations have been observed depending on whether CsA is characterized free in apolar solvents or bound in an aqueous complex with cyclophilin, its putative intracellular receptor (*16*−*21*). In both conformations, residues 7−11 form a compact antiparallel β-sheet, while the remaining residues form an open loop. The "free" conformation, derived from X-ray studies of CsA crystals grown in acetone and NMR experiments of the peptide dissolved in apolar solvents such as chloroform, is distinguished by the presence of a single cis peptide bond at MeLeu^9−MeLeu^{10} (the only cis peptide bond in the molecule) and four intramolecular hydrogen bonds (Val^5-NH−Abu^{2}CO, Abu^{2}NH−Val^{5}CO, Ala^{7}NH−MeVal^{11}CO, and D-Ala^{8}NH−MeLeu^{6}CO). In contrast, the "bound" conformation contains a trans peptide bond at MeLeu^9−MeLeu^{10} while the four amide protons participate in hydrogen bonds

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[1] Abbreviations: CsA, cyclosporin A; HBSS, Hanks' balanced salt solution; MDR, multidrug resistance; NEAA, nonessential amino acids; P-gp, P-glycoprotein; THF, tetrahydrofuran; TEER, transepithelial electrical resistance; TM, transport medium.

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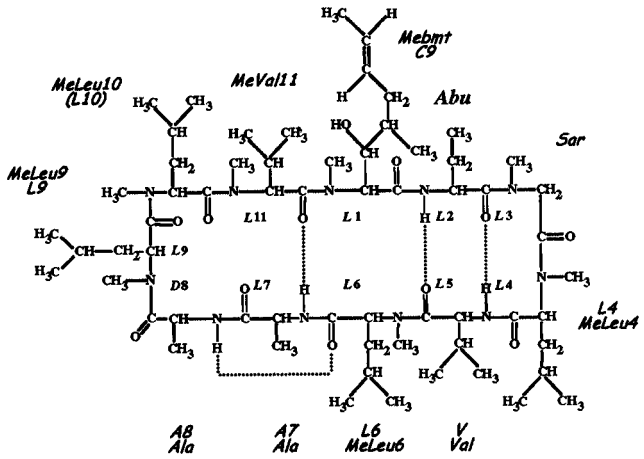

FIGURE 1: Structure of cyclosporin A derived from crystal studies. The dashed lines represent the four intramolecular hydrogen bonds (Val^{5}NH−Abu^{2}CO, Abu^{2}NH−Val^{5}CO, Ala^{7}NH−MeVal^{11}CO, and D-Ala^{8}NH−MeLeu^{6}CO) thought to be present in the cis (or "free") conformation.

with the protein partner, cyclophilin. In addition to these direct measurements, molecular dynamics studies in different solvents have suggested that these main-chain hydrogen bonds of CsA are intramolecular in nonpolar solvents, but form with water molecules in aqueous environments (*22*, *23*). One might predict that two different conformations, presumably the cis and trans, would result from the peptide satisfying these two discrete hydrogen-bonded states.

Both of the above CsA conformations appear stable at room temperature; however, there is now a large body of circumstantial evidence which suggests other conformational structures may exist in aqueous solution. Indeed, the work of several laboratories using NMR to glean structural information about CsA suggests that, in addition to the *free* and *bound* species, at least five other "slowly interconverting" conformers are in equilibrium in polar solvents such as DMSO, methanol, or methanol−water mixtures (*17*, *24*−*27*). With respect to the connection between CsA conformation and bioactivity, most of the efforts thus far have focused on the binding to cyclophilin. Therefore, it is currently unknown whether the capability of CsA to adopt different conformations plays a major role in the ability of the peptide to cross biological membranes to reach its site of action.

For small molecules with passive diffusion permeability characteristics, lipid solubility is the major factor for movement across cellular membranes with the degree of hydrophobicity correlating closely with the octanol−water partition coefficient of the molecule. For peptides, however, a rather poor correlation exists between membrane permeability and octanol−water partition coefficients. A better relationship is obtained when the energy of desolvation is considered as a principal determinant of transcellular transport. This desolvation energy is suggested to be directly correlated with the total number of hydrogen bonds the peptide is capable of forming with the bulk solvent (i.e., water) (*28*−*31*). According to this argument, the conformation of CsA and thus, the nature of these hydrogen bonds (i.e., whether they are intramolecular or intermolecular with water), should dictate the extent of membrane permeability.

During the course of earlier studies on CsA transport across Caco-2 cell monolayers (*12*), we discovered that the membrane permeability of the peptide was remarkably temperature dependent. For example, the rate of movement across the cells at 37 °C was typically about 80-fold greater than that measured at 4 °C. We hypothesized that this difference might reflect a shift in conformational equilibrium whereby, at lower temperatures, the predominant CsA species contains hydrogen bonds (via the four amide protons) with the bulk solvent, water. In contrast, at higher temperatures, we speculated that the major species contained intramolecular hydrogen bonds (i.e., the cis form), resulting in greater membrane penetration.

To examine the validity of this hypothesis, we have recently investigated the effect of temperature on both the transcellular movement of CsA across Caco-2 monolayers and on several physicochemical properties that should reflect the conformation or solvation state of the molecule. Physicochemical properties of the peptide studied included partition coefficients using solvent systems that have been reported to reflect hydrogen bonding potential, as well as aqueous solubility. Changes in the partial specific volume of CsA over the temperature range were observed by sedimentation equilibrium analytical ultracentrifugation. NMR spectra were also recorded in aqueous solution and, to our knowledge, represent the first such obtained for native CsA in aqueous solution. In the present manuscript, we describe results from these studies, suggesting that the solubility behavior of CsA may more closely resemble a traditional nonpolar solute than a peptide, and that membrane permeation is dictated by changes in the hydration shell rather than by changes in peptide intramolecular hydrogen bonds and conformation.

MATERIALS AND METHODS

[^{3}H]Cyclosporin A (11.1Ci/mmol), [^{3}H]propranolol (21.2 Ci/mmol), [^{14}C]salicylic acid (54 mCi/mmol), and [^{14}C]-mannitol (56 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Eagle's minimum essential medium (modified with Earle's salts, L-glutamine, and sodium bicarbonate) (MEM) was obtained from Fisher Scientific (Pittsburgh, PA). HEPES buffer, fetal bovine serum (FBS), MEM nonessential amino acid (100×) solution (NEAA), Hanks' balanced salt solution (HBSS), and 0.05% trypsin and 0.02% EDTA (10×) solution in HBSS were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A was kindly provided by Mr. Michael Green of Glaxo Research Institute. Deuterated solvents and buffer components were obtained from Cambridge Isotope Labs, Inc. (Andover, MA). The following extinction coefficient was used to determine aqueous concentrations of CsA: $\epsilon_{220nm} = 16\,500$ (M^{-1} cm^{-1}).

Caco-2 Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 75 cm^2 tissue culture flasks at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity using Eagle's MEM supplemented with 10% FBS and 1% NEAA (Culture medium). Cells were passaged at 80−90% confluency using phosphate buffered saline containing 0.02% EDTA and 0.05% trypsin. Cells were plated at a density of 80 000 cells/cm^2 on Costar (Cambridge, MA) polycarbonate membranes (Transwell cell culture inserts; diameter, 12 mm; mean pore size, 3.0μm) contained in Transwell clusters

(polystyrene, pyrrolidine-free, 24.5 mm diameter). Medium was changed every other day until day 17 and every day thereafter. Caco-2 cell monolayers were used between day 21 and 23 for all transport experiments. Cells between passage 51 and 73 were used throughout.

Membrane Permeation Studies. Culture medium bathing Caco-2 cell monolayers was removed and replaced with transport medium (TM; Hanks' balanced salt solution containing 25 mM HEPES and 25 mM glucose, pH 7.4). Following incubation at 37 °C for 30 min, transepithelial electrical resistance (TEER) values were measured using an epithelial voltohmmeter (World Precision Instr., New Haven, CT). Only those monolayers displaying TEER values greater than 350 $\Omega \cdot cm^2$ were used in studies. After an additional 30 min preincubation period at various temperatures (5, 10, 15, 20, 25, 29, 33, or 37 °C) in fresh TM previously equilibrated at those temperatures, transport medium in the apical (AP) compartment was removed. In all studies, compounds were added after equilibration of the system to the appropriate temperature.

For CsA, apical to basolateral transport was initiated by adding first 350 μL of unlabeled CsA (600 pmol), then 50 μL [^{3}H]CsA (0.3 μCi, 200pmol) to the apical compartment (total AP volume, 0.4 mL; final CsA concentration, 2 μM) of inserts held in transwells containing 1.5 mL of TM (basolateral compartment). For propranolol and salicylic acid, transport was initiated by adding 0.4 mL of a solution of either [^{3}H]propranolol (0.3 μCi) or [^{14}C]salicylic acid (0.04 μCi) to the apical compartment (final concentrations, 2 μM). Transwells were then incubated at various temperatures. At timed intervals (30, 60, 120, and 180 min), the inserts were carefully removed and placed in wells containing 1.5 mL of fresh TM, preequilibrated at the corresponding temperature.

For CsA sample processing, the insert was removed and 10 μL of 1 mM CsA added to the 1.5 mL of TM containing transported [^{3}H]CsA. Following a 1 h incubation (25 °C), the solution was removed via multiple pipetting with a single 200 μL disposable pipet tip, and placed (along with the tip) in borosilicate scintillation vials for liquid scintillation counting. To ensure total recovery of [^{3}H]CsA, 2 mL of TM containing 10 μM cold CsA in 10% DMSO was added to the empty wells for overnight incubation prior to collection and counting. For propranolol and salicylic acid sample processing, the basolateral solution was simply removed and placed in borosilicate scintillation vials for liquid scintillation counting (Earlier tests established that the nonspecific adsorption to plastic observed with CsA was not an issue with these two compounds.). In experiments comparing transport of CsA in the apical to basolateral direction versus that in the basolateral to apical direction, CsA addition and sampling was accomplished as described in Augustijns et al. (*12*) and a single 60 min time point measured.

In control experiments, the effect of temperature on tight junctions was assessed by studying the temperature-dependent flux of [^{14}C]mannitol (0.02 μCi) across Caco-2 monolayers. No significant differences were observed between the flux at 5 °C and at 37 °C. In all studies, TEER values did not significantly change over the time course of experiments under all temperature conditions.

Quantitation of CsA Transport. The amount of CsA crossing both membranes of the monolayer was determined by liquid scintillation (LS) counting of the appropriate solution using a Beckman LS 5801 spectrophotometer. All experiments were performed in triplicate with the results expressed as mean ± SD. "Transport" was defined and calculated as the percentage of compound appearing at the basolateral (or apical) side relative to the amount initially added to the apical (or basolateral) compartment (*12*). The average apparent permeability coefficients (P_{app}) in units of centimeters per second were calculated according to the following equation:

$$P_{app} = (dQ/dT)(1/A \cdot C_o) \quad (1)$$

where dQ/dT is the transport rate, C_o is the initial drug concentration on the donor side, and A is the surface area of the transwell insert (1 cm^2).

Determination of the Aqueous Solubility of CsA. A 10 mM stock solution of [^{3}H]CsA (2.5 μCi/mL) was prepared in THF. From this solution, 30 μL was added by Hamilton syringe directly into Pyrex glass test tubes equipped with Teflon caps. After evaporation of the THF, 3.0 mL of TM (5 °C) was added into the test tubes to obtain a suspension containing 100 nmol/mL CsA. To obtain saturated solutions, the suspensions were incubated overnight at 5 °C under gentle agitation. The tubes were then incubated overnight at various temperatures. Afterward, 600 μL of each solution/suspension was pipetted into a Corning, Inc. (Acton, MA) Spin-X centrifuge tube filter (0.45 μm CA filter) and centrifuged at 14 000 rpm for 2 min. The filtrate (200 μL) was placed (along with the tip) into borosilicate scintillation vials for liquid scintillation counting. The concentration of soluble CsA at various temperatures was calculated from a standard calibration set that was prepared by adding 30 μL aliquots of the 10 mM CsA stock solution directly into several borosilicate scintillation vials before counting. Values presented are averages of duplicate determinations.

Determination of the partition coefficient of CsA at various temperatures. (1) log $P_{isooctane/TM}$ and log $P_{heptane/ethylene\ glycol}$. [^{3}H]CsA in THF (adjusted to 1 mM with cold CsA) was added into Pyrex test tubes with Teflon caps. After evaporating the THF, either 25 mL of TM and 5 mL of isooctane, or 10 mL of heptane and 20 mL of ethylene glycol were added into the tubes. All solvents were mutually saturated before the partition experiment. The tubes were rotated overnight at various temperatures (6, 10, 15, 20, 25, 29, 33, and 37 °C). After centrifugation, aliquots from the upper layer (isooctane or heptane) were collected for liquid scintillation counting. Radioactivity in the bottom layer (TM or ethylene glycol) was calculated by subtracting counts in the upper layer from the total counts added. The log P was then calculated as

$$\log P_{phase1/phase2} = \log[(CPM_1 \cdot V_2)/(CPM_2 \cdot V_1)] \quad (2)$$

where phase 1 denotes the upper layer, phase 2 denotes the bottom layer, CPM_1, and CPM_2 represents the counts per minute in the upper and bottom layer, respectively, and V_1 and V_2 are the volumes of these layers.

(2) log $P_{octanol/TM}$. A solution of [^{3}H]CsA was prepared in Pyrex test tubes containing 5 mL of octanol. After addition of 25 mL of TM, tubes were rotated overnight at various temperatures. Following centrifugation, aliquots were taken from both the octanol layer and the TM layer for liquid

scintillation counting. The log *P* was calculated according to the equation above.

Proton-NMR Experiments. The CsA used in studies throughout this manuscript was analytically pure (>95% by reversed-phase HPLC). However, for NMR studies in aqueous solution, trace contaminants in the solid material complicated sample preparation due to their increased water solubility relative to CsA. The sample was, therefore, cleaned by first dissolving in $CHCl_3$, extracting multiple times with distilled H_2O and finally, drying under a stream of nitrogen gas. Solid CsA, dried on the walls of a glass vial, was taken up into various aqueous media by gentle rocking overnight at 4 °C followed by centrifugation (RCF = 16 000/10 min) and transferred into 8 mm susceptibility-matched NMR tubes (Shigemi Inc., Tokyo, Japan). Solvent pH was adjusted with concentrated DCl or NaOD before CsA dissolution. Peptide concentrations were estimated by comparison of integrated signal intensities to an internal trimethylsilylpropionate-d_4 (TMS) standard. Field-frequency lock was achieved through incorporation of 5% D_2O in the buffer. ^{1}H NMR spectra were acquired on a Varian Assoc. Unity$^+$-500 MHz. NMR spectrometer (Palo Alto, CA) equipped with an 8 mm triple resonance HCN probe (Nalorac, Martinez, CA). Water suppression was accomplished using either Jump-Return (*32*) pulses with the excitation maximum set to 8 ppm, or optimized WATERGATE (*33*) pulses. Spectra were acquired by summing 200−10000 transients having a spectral width of 8000 Hz and processed using FELIX 2.35 (MSI, La Jolla, CA) software. Baselines were improved by forward linear prediction of the first three points, with any residual water signal suppressed by convolution difference processing. A line-broadening constant of 3 Hz was used.

Spectra generated from such samples were much improved, showing minimal contamination by other components. Concentrations of saturated CsA in pure water were ~180 μM at 4 °C, as determined by UV and NMR spectra. Heating of these solutions caused precipitation of CsA, observable as Raleigh scattering. However, CsA readily redissolved when temperatures were lowered. More importantly, NMR and optical spectra were identical after multiple rounds of temperature cycling between 5 and 40 °C. Thus, it appeared that CsA was not supersaturated at these concentrations. Attainable concentrations in more physiological buffers, such as transport media, were lower (<50 μM), but still adequate for obtaining ^{1}H NMR spectra.

Analytical Ultracentrifugation. Sedimentation equilibrium analytical ultracentrifugation on CsA was performed using a Beckman XL-A centrifuge (Beckman Instruments, Inc., Palo Alto, CA) with two-channel 12 mm charcoal-filled Epon centerpieces. Runs were performed at 40 000 rpm at various temperatures with scans taken at 220 nm at 1 h intervals. Equilibrium was judged to be achieved by the absence of change between plots of several successive scans after approximately 48 h. Each 180−200 mL sample of CsA (15 μM) in 24 mM Tris, 24 mM glucose, and HBSS (pH 7.4) was centrifuged against 250 mL of the equivalent buffer blank. Solvent density was determined empirically at each temperature using a Mettler DA-110 density/specific gravity meter calibrated against water. Data sets were obtained as radial distance versus absorbance and were analyzed by the Beckman/Microcal Origin nonlinear regression software package using multiple iterations of the Marquardt−Levenberg algorithm (*34*) for parameter estimation. Absorbance values as measured in the sedimentation gradient were taken to represent the concentration of CsA at each radial position. Best fits were obtained with an ideal monomer model using the known molecular weight and varying the hydrodynamic factor. This model can be described mathematically as follows:

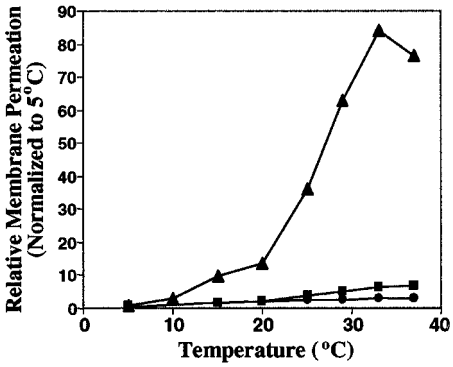

FIGURE 2: Effect of temperature on transepithelial flux of CsA (▲), propranolol (●), and salicylic acid (■). The amount of each compound appearing in the basolateral compartment at various temperatures was normalized to the amount crossing the monolayers at 5 °C. Values shown are averages of three determinations.

$$c_r = c_o e^{MH(r^2 - r_o^2)} + E \quad (3)$$

$$\ln c_r = \ln c_o + MH(r^2 - r_o^2) + \ln E \quad (4)$$

where c_r is the concentration at radius r, c_o is the concentration at radius r_o, M is the molecular weight, H is the hydrodynamic factor, and E is a baseline offset. The hydrodynamic factor is comprised of experimental variables pertaining to the solution properties and is defined by

$$H = \frac{(1 - \bar{\nu}p)\omega^2}{2RT} \quad (5)$$

where $\bar{\nu}$ is the partial specific volume, p is the solvent density, ω is the angular velocity (2π·RPM/60), R is the ideal gas constant (8.314 J·K^{-1} mol^{-1}), and T is the temperature in Kelvin.

RESULTS

Permeation of CsA Through Biological Membranes. Work in our laboratory using Caco-2 cell monolayers (*12*) has supported the views of others (*35*−*38*) that CsA crosses biological membranes via passive diffusion. However, during the course of our studies, we noted a remarkable increase in the apical to basolateral permeation rate (80-fold) of the cyclic peptide as the temperature was increased from 5 to 37 °C. The magnitude of this temperature-dependent enhancement of permeability would not be anticipated for a molecule undergoing simple passive diffusion.

Therefore, we have extended that original study to include a range of temperatures, comparing the apical to basolateral movement of the CsA peptide (2 μM) with that of two small molecules, propranolol and salicylic acid. These two model compounds are also known to cross biological membranes by passive diffusion (*39*, *40*). Figure 2 shows the apical to basolateral migration for the three compounds over a range

Table 1: Apparent Permeability Coefficients (P_{app}) for CsA (5 μM) Migration Across Caco-2 Monolayers in *Apical* to *Basolateral* and *Basolateral* to *Apical* Directions

	apical to basolateral			basolateral to apical		
	P_{app}[a]	SD[b]	enhancement[c]	P_{app}	SD	enhancement
5 °C	0.027	0.001		0.035	0.008	
25 °C	0.478	0.081	17.7	1.20	0.197	34.29
37 °C	3.83	0.203	142	7.35	0.489	213.0

[a] P_{app} = Apparent permeability coefficients for the 1 h transport study were calculated as described in the Material and Methods. [b] SD = standard deviation from a set of quadruplicates. [c] Enhancement is defined as the increase in permeability relative to 5 °C.

of temperatures, with the resultant P_{app} values (eq 1) normalized to those obtained at 5 °C and expressed as *Relative Membrane Permeation*. This figure clearly illustrates the dramatic differences in membrane permeability for CsA as a function of temperature. Thus, while propranolol and salicylic acid realized modest gains (3- and 7-fold, respectively) in permeability with increasing temperature, we observed an 80-fold increase in CsA permeability as incubation temperatures were increased from 5 to 37 °C.

We have shown previously that Caco-2 cells possess an MDR-related P-gp pump, presumably located at the apical surface, capable of recognizing CsA as a substrate (*12*). Since an active pump should be relatively quiescent at 5 °C, this polarized efflux system has the effect of under-representing the magnitude of the difference in membrane permeability of CsA when apical to basolateral transport is measured at 5 vs 37 °C. The true impact of temperature on CsA transport is illustrated better when both apical to basolateral and basolateral to apical permeability rates are determined at different temperatures using concentrations of CsA (5 μM) that should be closer to saturating the active transporter. Table 1 shows the P_{app} values for CsA movement in both directions across Caco-2 monolayers at 5, 25, and 37 °C. Inspection of values for the increase in permeability at 37 °C relative to 5 °C shows a 142-fold increase for the apical to basolateral direction and a more than 200-fold increase in the basolateral to apical direction (which includes the P-gp contribution to transport). The magnitude of these effects of temperature on CsA permeability cannot be explained solely by temperature-induced changes in membrane fluidity (*41*) or solvent viscosity and suggests that more significant events are responsible.

Temperature-Dependent Solubility. Next, we examined the solubility of CsA in transport medium over the same temperature range as above. For most molecules, solubility increases with increasing temperatures due to changes in the relative strengths of solvent−solvent, solvent−solute, and solute−solute interactions. However, for CsA in Caco-2 transport medium, we found that the solubility of this peptide was inversely related to temperature (Figure 3). While the solubility of CsA at 5 °C was 72 μM, the measured solubility decreased by more than an order of magnitude to 5 μM at 37 °C. This finding agrees with earlier solubility data obtained in water or citrate buffers at pH 1.2 or 6.6, respectively (*42*).

These solubility results are consistent with the hypothesis that at lower temperatures a solvent (i.e., water) hydrogen-bonded conformation predominates, whereas at higher temperatures, an internally hydrogen-bonded (hydrophobic) conformation is preferred.

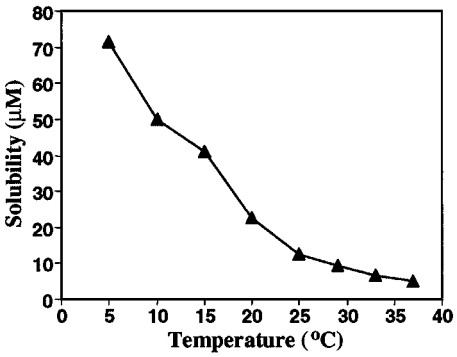

FIGURE 3: Temperature dependence on the solubility of CsA in transport medium (pH 7.4) between 5 and 37 °C.

Partition Coefficients as an Indicator of Hydrogen Bonding/Hydration State. The partition coefficients for CsA were measured at temperatures identical to those employed in the above transport and solubility studies. More importantly, these studies were conducted using solvent systems that reflect the hydrogen-bonding potential of peptides. Chikhale and co-workers (*28*) have suggested that, for peptides, hydrogen-bonding potential may be estimated using either the measured heptane/ethylene glycol partition coefficient or the calculated isooctane/octanol partition coefficient (obtained as the difference between partition coefficients using the octanol/transport medium partitioning system and isooctane/transport medium partitioning system). The temperature dependence of both the calculated isooctane/octanol partition coefficient and the heptane/ethylene glycol partition coefficient is illustrated in Figure 4 and shows that, as temperature increases, CsA preferentially partitions into the non-hydrogen-bonding solvent (isooctane, heptane).

These observations would agree both with changes in peptide hydrogen bond status (internal vs external) as a consequence of conformational shifts and the "hydrophobic effect" (*43*−*45*), which describes the behavior of nonpolar solutes in aqueous media.

Proton NMR Studies of CsA. The ^{1}H NMR resonances of the four amide protons of CsA have been assigned in various organic solvents including chloroform, benzene, and tetrahydrofuran (*46*). Whereas in solvents of higher polarity (e.g., DMSO), multiple backbone conformations are directly visible in the NMR spectra, in tetrahydrofuran (THF-d_8) CsA exists as one major conformer. In our studies, a suite of two-dimensional ^{1}H NMR spectra of 1 mM CsA in THF-d_8 was acquired and assigned, and the structure was identified. The sample was found to be clean with spectra identical to those reported previously. Resonances of the four amide protons of the single major conformer are apparent (Figure 5). Only resonances from one molecule type were observed, with no unexplained peaks present.

The NMR spectra of CsA in pure water at 15 °C were more complex, as illustrated in Figure 6A. Inspection of the spectra, obtained in three distinct pH environments, revealed the presence of at least eight amide resonances in water (although there are only four amide protons). This observation can be explained only by the existence of multiple conformers in aqueous solution. Furthermore, it is clear that at least two conformers of CsA exist in water (relative ratios of 2:1) that account for >80% of the total population. No concentration-dependent changes in the NMR spectra were

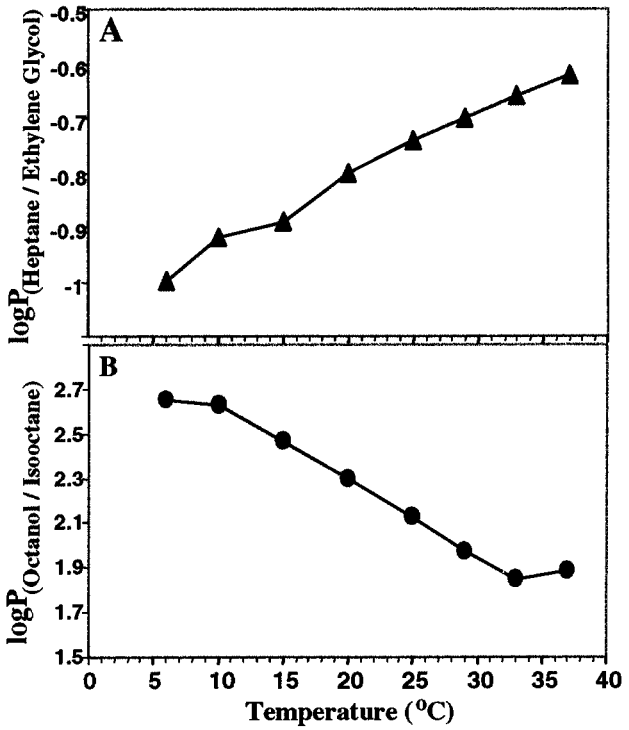

FIGURE 4: Effect of temperature (5−37 °C) on the measured partition coefficient of CsA in the heptane/ethylene glycol solvent system (A), and the calculated partition coefficient in the theoretical octanol/isooctane solvent system (B). Partition coefficients obtained in these solvent systems were shown previously to be predictive of the hydrogen-bonding potential of peptides (*28*). Values shown represent the logarithms (log *P*) of the mean of three determinations. The octanol/isooctane partition coefficient was obtained by subtracting the log *P* values for CsA in the isooctane/TM system from the log *P* values measured for CsA in the octanol/TM system.

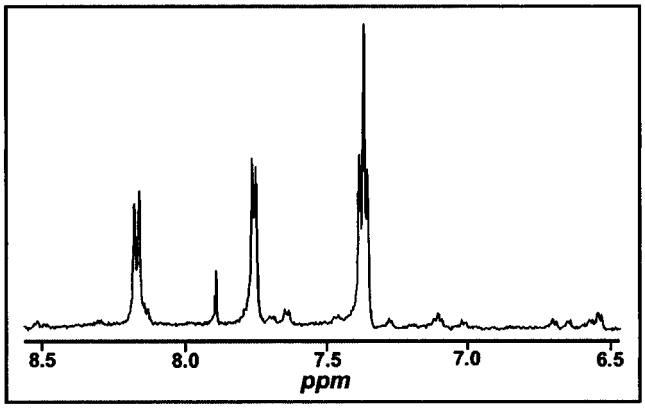

FIGURE 5: ^{1}H NMR spectrum of CsA (1 mM) in THF-d_8. Only the amide resonances between 6.5 and 8.5 ppm are depicted. Four resonances from the major conformer are evident, as well as several resonances from minor conformers in slow equilibrium at the NMR time scale.

observed at CsA concentrations ranging from 3 to 180μM (data not shown). Thus, results obtained at higher concentrations with good signal-to-noise may be reasonably extrapolated to the lower concentrations used in permeability studies. In addition, self-aggregation effects were not evident over this concentration range.

The intramolecular hydrogen bonding frequently observed for some amide protons of certain CsA conformers has been evoked to explain various functional behaviors (e.g., binding to cyclophilin) of the cyclic peptide (*26*). The number of hydrogen bonds a peptide is capable of forming with water has also been hypothesized to correlate with its ability to cross cellular membranes. Therefore, it was of interest to investigate the nature of the hydrogen bonds within the CsA conformer population observed in water. The measurement of chemical proton exchange rates between amide protons and water is one of the most sensitive indications of hydrogen bonding (*47*) and is theoretically well understood (*48*). These exchange rates are both pH- and temperature-dependent, with different rates easily observable as preferential line broadening or chemical shift changes of specific amide resonances as compared to others. Furthermore, no charge titration effects are possible for CsA. However, careful examination of the amide resonances for CsA in the three different pH environments shown in Figure 6A did not yield evidence for specific, strong intramolecular hydrogen bonding in the major CsA conformers in water.

The effect of temperature on NMR spectra was also investigated (Figure 6B). Spectra were obtained in an interleaved fashion where the temperature was changed according to the following sequence: 5, 15, 25, 35, 30, 20, 10, and 5 °C. Again, no significant, selective line broadening was observed. The temperature dependence of the amide chemical shifts was greater, however, for the two resonances at higher field (7−8 ppm), indicating less protection from solvent exchange than the other amide resonances at lower field. Further examination of the spectra reveals no dramatic changes in the populations of the various CsA conformers over this temperature range as well, although a weak resonance appears at 8.65 ppm at temperatures between 15 and 30 °C, reaching maximal intensity at 22 °C.

The above NMR data show that various conformations of CsA exist in aqueous solutions; however, the equilibrium between the two major species is not affected by temperature variations. The only perturbation of these equilibria was the temperature-dependent alteration of a minor and, as of yet, unidentified species. These NMR data suggest that the observed dramatic temperature-dependent changes in CsA permeability do not originate from temperature-induced changes in peptide backbone conformation. The studies described below were conducted with the aim of examining the hydration state of the peptide as a function of temperature.

Sedimentation Equilibrium Analysis of CsA. Initially, CsA (15 μM) was examined by sedimentation equilibrium analytical ultracentrifugation at various temperatures to determine if the peptide was aggregating or self-associating in our aqueous buffer system. Data analysis using an ideal monomer model and predicted values for partial specific volume (*49*, *50*) suggested that CsA behaved in a markedly nonideal manner. In fact, the data indicated that the apparent CsA molecular mass decreased from its expected compositional molecular mass (1202 Da) to about 500 Da as temperature was increased from 4 to 37 °C. This phenomenon was found to be fully reversible as lowering the temperature from 37 to 4 °C resulted in the "restoration" of the expected molecular mass. Mass spectral analysis confirmed that the CsA remained intact following these studies. Since this reversibility rules out degradation of the peptide, the best explanation appears to be that CsA is exhibiting aberrant hydrodynamic behavior as temperature is varied over this range.

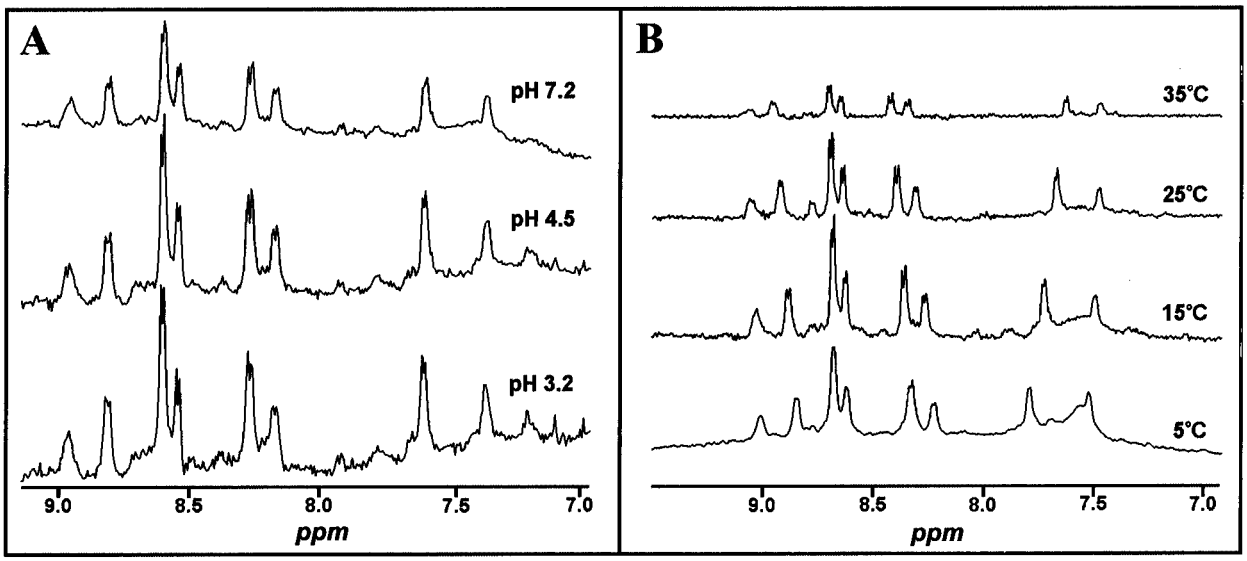

FIGURE 6: ^{1}H NMR spectra of CsA (~180 μM) in pure water (A) at 15 °C and various pH values as indicated. (B) pH 7.3, at various temperatures as indicated.

The hydrodynamic factor is defined in eq 3 (see Materials and Methods) and contains five parameters that affect the hydrodynamic contribution to sedimentation. The partial specific volume is the only one of these parameters which was not empirically determined, experimentally controlled, or constant. Reanalysis of the sedimentation data using the ideal monomer model, but with the molecular mass held constant at 1202 Da and allowing the hydrodynamic factor to float, resulted in fits of equivalent quality to those initially obtained. This hydrodynamic factor was then algebraically deconvoluted to yield values for the partial specific volume of CsA. Components of the partial specific volume of a molecule include both its mass and waters of hydration. Partial specific volume is inversely proportional to the extent of the hydration shell (*49*). As shown in Figure 7, these values were found to change significantly over the experimental temperature range, varying from 0.83 mL/g at 4 °C to 0.91 mL/g at 37 °C. This result is in sharp contrast to the normally expected variation of partial specific volume with temperature. The most logical interpretation of this observation is that the hydration state of CsA changes significantly as a function of temperature.

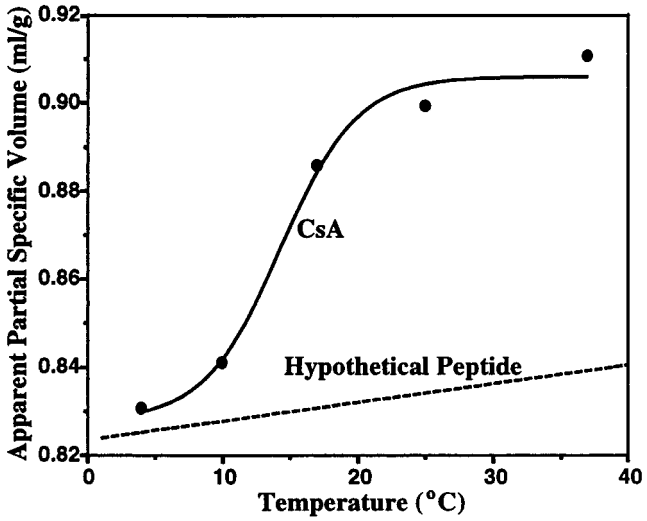

FIGURE 7: Change in CsA apparent partial specific volume over temperature. Data from multiple data sets (4−8) for each experimental temperature (4, 10, 17, 25, and 37 °C) were fit for partial specific volume using an ideal monomer model. Average partial specific volume values (●) were plotted versus temperature. The dashed bottom line represents the slope of a predicted linear change in partial specific volume for a hypothetical peptide over the same temperature range using the method of Durshlag (*9*).

DISCUSSION

The physical features responsible for the unusual behavior of cyclosporin A have been the subject of intensive investigation since its synthesis was first described (*13*, *15*). However, despite an enormous amount of effort, relatively little is known today about the various conformer options available in aqueous media and how they relate to the ability of this peptide to permeate biological membranes. This dearth of information is due, in large part, to the extreme hydrophobicity of the molecule which has necessitated that most of the characterization of CsA be conducted in either organic solvents or while the peptide was bound to cyclophilin. The goal of our research was to examine the relationship between molecular conformation and membrane permeability of the native peptide under physiologically relevant (i.e., aqueous buffer) conditions. To accomplish this objective, we examined the physicochemical characteristics of CsA under the same conditions in which we quantitated its membrane permeation.

We began these studies with the belief that our initial observations on the effect of temperature on CsA permeability may be explained by a theory advanced by Burton and co-workers (*28*−*31*) in the early 1990s and widely cited thereafter in peptide literature. Their work using sequentially modified model peptides suggested that, for this class of molecules, the desolvation energy required to disrupt hydrogen bonds formed with water is the major determinant for membrane permeability and that this energy is not favorably offset by the introduction of lipophilic amino acid side chains. Knowing that CsA can exist in multiple conformations differing by the nature and number of intramolecular hydrogen bonds formed, it seemed reasonable to propose that the transition between these conformers was

involved in the temperature effect. Thus, we set out to test the hypothesis that, at *low temperatures*, CsA exists predominantly in the trans conformation in which the four amide protons hydrogen bond with water molecules, increasing the energy required for desolvation and thereby reducing membrane permeability. Conversely, as temperature is increased, we expected to document a shift in conformational equilibrium such that at 37 °C there would be the almost exclusive existence of the cis conformer containing intramolecular hydrogen bonds − a structural motif quite amenable to membrane permeation. Increasing our level of confidence in this model was the fact that, over the years, indirect evidence has accumulated for the existence of at least several discrete "slowly interconverting" configurations in equilibrium (*16*, *17*, *24*−*26*).

Experiments in which we compared the membrane permeation of CsA to model compounds for passive diffusion (Figure 2) clearly showed the unique temperature dependence of the cyclic peptide. Propranolol and salicylic acid were selected due to their relatively high degree of lipophilicity and permeability in the Caco-2 model (*39*), and because the membrane permeation characteristics of salicylic acid, like CsA, have been speculated to involve a conformation featuring an intramolecular hydrogen bond (*51*). The pattern of temperature dependence for CsA continued during solubility studies that showed the peptide to be an order of magnitude more soluble at 5 than at 37 °C (Figure 3). When considered alone, this result could be interpreted as the peptide adopting, at higher temperatures, the cis conformation possessing intramolecular hydrogen bonds and greater hydrophobic character.

On the basis of the above information, we sought to measure the hydrogen-bonding status (and by inference, conformation) of the peptide at different temperatures. We accomplished this objective by measuring partition coefficients using solvent systems previously shown to allow for estimation of hydrogen bond potential (*28*). Again, a dramatic temperature effect was observed with CsA partitioning, in linear fashion as a function of temperature, into the non-hydrogen-bond donating solvent.

Thus, membrane transport, aqueous solubility, and partitioning studies all confirmed a temperature-dependent change in the physical properties of CsA. It was tempting to evoke the Burton hypothesis as the explanation for these observations and conclude that CsA simply obeys that model. However, we believed that these data could also be interpreted in terms of CsA simply becoming more hydrated at lower temperatures through the hydrophobic effect documented for non-polar molecules in aqueous media (*43*−*45*). Indeed, the solubility behavior of CsA matches that described for nonpolar solutes over the temperature range tested. This alternative model would not require a change in peptide conformation or hydrogen bond status.

In an attempt to resolve the issue, we employed NMR to examine the conformation of CsA under various solution conditions. On the basis of the known CsA structures, the four amide protons that mark the differences in these structures were the focus of our experiments. In the past, solubility limitations had restricted the type and amount of structural data obtained for native CsA in aqueous solutions. However, recent improvements in NMR technology, especially methods for suppression of water resonance, provided us the opportunity for NMR spectral acquisition of CsA in biologically relevant buffers. The NMR spectra shown in Figure 6 represent, to the best of our knowledge, the first such measurements for native CsA in aqueous solution. These results demonstrated, as predicted by earlier researchers, that multiple conformers of CsA exist in water as eight distinct amide proton resonances were observed. However, the ratios of these conformers did not change significantly as a function of temperature, pH, or salt. Therefore, the temperature dependence of CsA cannot be explained by a conformational change involving the four amide proton hydrogen bonds as we speculated in our original hypothesis. Other CsA resonances, such as the *N*-methyl groups and other resolved side-chain groups, were also observed not to change with temperature, pH, or buffer components.

While the NMR data appeared to rule out a role for the predicted conformational change in the behavior of CsA, the sedimentation equilibrium analysis provided compelling evidence that the temperature-induced modulation in membrane permeation is the result of changes in the hydration state of the molecule. As illustrated in Figure 7, the partial specific volume of CsA increases significantly at higher temperatures. Given the conservation of peptide mass, this change must be due to a decrease in the number of water molecules in (or the strength of) the surrounding hydration shell. Such an effect would, in turn, result in lower desolvation energies and thus increased membrane permeability, increased partitioning into nonpolar solvents, and lower aqueous solubility. These waters of hydration arrive at equilibrium between being part of the bulk solvent and being hydrogen bonded to CsA. Clearly, temperature influences the dynamics of the CsA hydration equilibrium.

Therefore, it is our hypothesis that the temperature-induced changes in membrane permeation of CsA are the result of alterations in the hydration state of the molecule brought about by either (1) a reordering of water molecules about the peptide surface or (2) changes in hydrogen bonding, between water and CsA, that are not discernible by current technologies. In the first model, a smaller hydration shell envelops the molecule at higher temperatures, resulting in a lower desolvation energy and thereby promoting partitioning into cellular membranes. Conversely, as the temperature is lowered, there is a coordinated reordering of water molecules about the peptide, producing a more extensive hydration shell that has the effect of making CsA more soluble in water but severely impeding its ability to penetrate cells. In this case, desolvation is defined as the energy cost for this reordering of waters of hydration around the peptide solute. In support of the second model in which hydrogen-bonding events are evoked are several molecular dynamics studies reporting the simulated hydrogen bonding of CsA in water (*23*, *52*). These investigators predicted various degrees of hydrogen bonding for other CsA atoms, including backbone carbonyls and the 1-MeBmt side-chain hydroxyl. Thus, at lower temperatures, relatively strong hydrogen bonds exist between water and CsA, resulting in its increased solubility and decreased propensity for membrane penetration. In this case, desolvation is defined as the energy cost for the formation or disruption of hydrogen bonds between CsA and water.

While the NMR data clearly show that *major* conformers of CsA do not change in population sufficiently to explain the temperature dependence on membrane permeation, it is

conceivable that a *minor* species is responsible for the observed differences. In one plausible scenario, an equilibrium could be established among the various conformers such that very small amounts of the most permeable structures are produced (perhaps through some interaction with the membrane) then rapidly cleared via permeation through the membrane, making their measurement impossible with existing techniques. However, a minor species could not be responsible for the gross change observed in the measured partial specific volume of the peptide. Therefore, we favor the interpretation that a significant change in the hydration shell occurs over the temperature range inspected.

In conclusion, in this manuscript, we have documented the first direct experimental evidence obtained with CsA in an aqueous environment that addresses the question of the mechanism(s) behind the unusual membrane permeation characteristics of the peptide. The data support the hypothesis that hydration shell effects are the primary drivers behind our observations. However, with the exception of the amide and resolved, but unidentified side chain, protons, we could not directly examine specific hydrogen bonding within CsA. Thus, the phenomena described above could also be interpreted as consistent with the Burton hypothesis for peptide movement across membranes, and we cannot rule out that possibility at this time. A highly significant finding in light of the focus in CsA literature on cis and trans isomerizations is the fact that the temperature-induced phenomena do not appear to be related to changes in peptide backbone conformation.

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