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Interactions of Cyclosporines with Lipid Membranes as Studied by Solid-State Nuclear Magnetic Resonance Spectroscopy and High-Sensitivity Titration Calorimetry

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ABSTRACT: Cyclosporin A (CyA) interacts with lipid membranes. Binding reaction and membrane location of CyA and analogs were examined with ^{2}H-NMR, high-sensitivity isothermal titration calorimetry (ITC), and CD spectroscopy. Effects of CyA and charged analogs on the phosphocholine head group and on the membrane interior were investigated using selectively deuterated phospholipids. Incorporation of cyclosporin generated small disordering of the lipid acyl chains. Binding of CyA and neutral and positively charged analogs to lipid membranes showed endothermic heats of reaction between $+5.9$ and $+11.3$ kcal/mol, whereas enthalpy of binding was close to zero for the negatively charged derivative. Binding constants of cyclosporines to liposomal membranes were in the range of $K_P = 1650–5560\ M^{-1}$ depending on the cholesterol content. ^{2}H-NMR provides evidence that CyA is essentially located in the interior of the bilayer membrane. For the charged analogs an additional interaction occurs at the head group level, placing the polar groups of these CyA analogs in the vicinity of the phosphocholine dipoles. The association of CyA and its analogs is accompanied by a positive enthalpy change, which is overcompensated by positive entropy changes. Binding of CyA to lipid membranes thus follows the classical hydrophobic effect, which is in contrast to many other peptide-lipid binding reactions. J Pharm Sci 91:856–867, 2002

Keywords: cyclosporines; lipid membranes; solid-state nuclear magnetic resonance spectroscopy; titration calorimetry

INTRODUCTION

Cyclosporin A (CyA) is the active component of Sandimmun®, an efficient and clinically used immunosuppressive agent. It is the drug of choice in transplantation medicine and the treatment of auto-immune diseases. The cyclic peptide is composed of 11 amino acids (seven of them are N-methylated and two amino acids are rather uncommon ones). Two conformations have been reported for CyA: one for pure CyA, the other for CyA bound to immunophilin or calcineurin.[1] The binding to calcineurin is the basis for the pharmacological action of CyA.[2]

A serious side effect of CyA is a reversible nephrotoxicity, the mechanism of which remains unclear up to now.[3] The high lipophilicity of CyA has led some authors to suggest that a perturbation of membrane functions by incorporated CyA could evolve after treatment with CyA[4] and that

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immunosuppression is also achieved by partitioning of CyA into the bilayer. Some evidence for the direct action of CyA on the properties of lymphocyte membranes was published.[5] Other reports, investigating the interaction of CyA with model membranes, mention an increase in membrane fluidity upon addition of CyA at temperatures below the main phase transition, but an increased order at temperatures above the transition temperature.[6,7] It was recently argued, that CyA might change lipid domain morphology as demonstrated by lipid monolayer studies[8] and may also inhibit membrane fusion under certain circumstances.[9]

In pharmaceutical technology lipid membranes are used as drug carrier systems in the form of liposomes. From a pharmaceutical point of view liposomes can be regarded as being nontoxic and biodegradable carriers because the components of this carrier are mostly made of membrane compounds found in mammalians. Intravenously administered liposomes also appear not to accumulate in the kidney.[10]

Because of the high lipophilicity of CyA more than 30 published studies tried to take advantage of liposomes as carrier systems for CyA to lower the side effects in the kidney during immunsuppressive therapy, as liposomes tend to avoid kidney tissue and tend to accumulate in the spleen, one of the assumed target organs for CyA (see ref. 10 for a review). One other reason using liposomes is the attempt to avoid Cremophor EL, the excipient in the commercial formulation showing intrinsic renal side effects in several studies.[11] Obviously, the exact binding place and the extent of CyA binding to the liposomal membrane are of interest for optimization of liposomal formulations, as CyA tends to "leak" out of the liposomes in short time.[12] In addition, the lipid composition regarding fatty acid chains and especially the lipid head group of the employed liposomes appears to be important.[13] Because CyA binds to blood components, thereby changing pharmacokinetic variables such as clearance,[14] binding characteristics of CyA to liposomal membranes are also of clinical interest.

The purpose of this study was to investigate the interaction of CyA and three different analogs (hydroxylated CyA-OH, cationic CyA-NH_3^+, and anionic CyA-SO_3^-; cf. Figure 1) with phospholipid membranes. Deuterium NMR, in combination with selectively deuterated phospholipids, was used to identify the membrane location of CyA. Isothermal titration calorimetry provided the heat of reaction, the binding isotherm, and the water–membrane partition coefficient. CD spectroscopy was used to measure CyA concentrations in solution as the molecule does not contain UV-detectable amino acid residues.

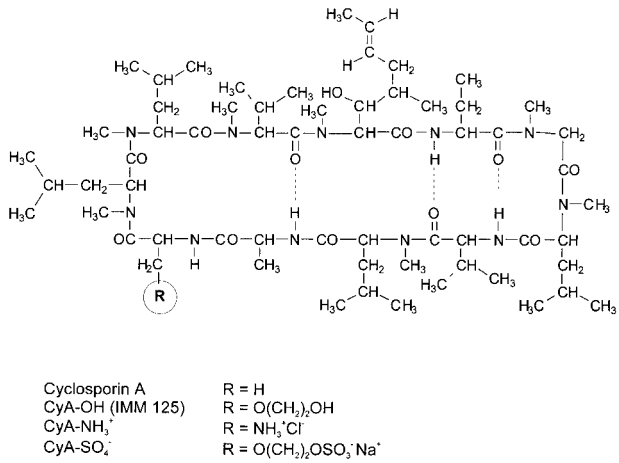


Figure 1. Chemical structure of cyclosporin A and its derivatives.

MATERIALS AND METHODS

Drugs and Chemicals

Cyclosporin A (CyA), CyA-OH (SDZ 216-125 = IMM-125), CyA-NH_3^+ (SDZ 213-964), and CyA-SO_4^- (SDZ 218-797) were generously provided by Novartis Inc., Basle, Switzerland. Their chemical structure are given in Figure 1. Cholesterol was obtained from Sigma (Buchs, Switzerland) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL).

POPC was selectively deuterated at the α- and β-segment of the choline head group as described previously.[15] 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine ([9′,10′-$^2H_2$]POPC) was prepared according to ref. 16.

If not stated otherwise, the experiments with CyA and CyA-OH were performed in pure water. The charged derivatives CyA-NH_3^+ and CyA-SO_4^- were measured in 10 mM Tris buffer at pH = 7.4. Deuterium-depleted water was used for the ^{2}H-NMR measurements.

Liposome Preparation

Liposomes composed of POPC were made by drying a lipid solution in dichloromethane (with

appropriate addition of cholesterol dissolved in dichloromethane/ethanol = 2:1) under a stream of nitrogen and drying overnight *in vacuo*. Water or Tris buffer was added, and the mixture was vortexed until a homogeneous multilamellar liposome suspension was achieved. Small unilamellar liposomes (SUV) with a diameter of about 30 nm were prepared by sonication of this suspension for 45 min at 10°C (MSE 100 Watt London, England), whereas 100 and 400 nm unilamellar liposomes were prepared by extrusion through polycarbonate filters.[17] Size measurements of the liposomes were made by dynamic laser light scattering with a ZetaSizer III (Malvern, UK).

Solubility Measurements

Well-defined amounts of cyclosporin A (10–50 mg) or CyA-OH were dissolved in 1 mL ethanol. The clear solutions were added slowly and under constant stirring to 1 L of water or 1 L NaCl (0.164 M). The final concentration of ethanol was thus $\approx 0.1\%$. At concentrations of less than 30 mg cyclosporin per liter, the solutions remained optically clear at room temperature, at higher concentrations they appeared opalescent. All solutions were cooled to 4°C for about 12 h. The optically clear solutions were filled in the quarz cuvette of a fluorimeter and measured at right angle to the excitation beam (excitation wave length $\lambda = 350$ nm, measuring wave length $\lambda = 350$ nm). The temperature was increased in steps of 1°C. The limit of monomer solubility was defined as the first occurrence of light scattering.

CD Measurements

Circular dichroism experiments were performed using a Jasco J-720 Spectropolarimeter (Jasco, Japan). Cyclosporin concentrations were determined using a mean residue ellipticity $\Theta = -2.64 \pm 0.03 \cdot 10^4$ deg cm^2 d mol^{-1} at $\lambda = 226$ nm.

NMR Measurements

POPC stock solution (1–1.5 mL; 20 mg/mL) in dichloromethane was pipetted into the NMR sample tube, and the solvent was evaporated under nitrogen. The dry lipids were weighed, and the appropriate volume of a cyclosporin stock solution (dichloromethane) was added according to the desired molar ratio. The solvent was removed and, after another weighing, the molar ratio of cyclosporin to POPC could be calculated.

For ^{2}H-measurements the lipid mixtures were dispersed in 70 μL deuterium-depleted water to form coarse, multilamellar liposomes.

Solid-state NMR spectra were recorded on a Bruker MSL 400 spectrometer operating at 61.4 MHz for ^{2}H. A quadrupole echo sequence with ~ 5 μs 90° pulses, and a pulse spacing of 40 μs was used.[18]

Titration Calorimetry

Thermodynamic effects were measured with Microcal MC-2 and Microcal VP high-sensitivity titration calorimeters (Microcal Inc., Northhampthon, MA).[19] The probe solutions were degassed under vacuum prior to use. Data were acquired and analyzed by software developed by Microcal. Partition constants, and reaction enthalpies were measured by lipid-into-peptide titrations:[20] the liposome suspension (about 20 mM in lipid) was injected in aliquots of 10 μL into the sample cell filled with cyclosporin solution (about 15 μM). Each injection produced an endothermic heat of reaction which was determined by integration of the heat flow tracings.

RESULTS

Solubility and Aggregation of CyA and CyA-OH in Aqueous Solution

The noncharged cyclosporines are only sparingly soluble in water. Well-defined aqueous solutions can be prepared by dissolving the peptides in ethanol and by slowly stirring the ethanolic solution into water (final ethanol concentration 0.1%). At low concentrations (< 15 μM) CyA and CyA-OH are monomeric in solution (at 25°C) as evidenced by monolayer surface activity measurements (data not shown); at higher concentrations the peptides form aggregates and precipitate. A critical micellar concentration (CMC) of CyA of ~ 30 μM was determined previously by light scattering and dye adsorption (at 20°C; PBS buffer),[21] but no temperature dependence was reported. In the present study, clear solutions of different peptide concentration were prepared at 4°C and were characterized as a function of temperature with light scattering. At a well-defined temperature, a first, weak increase in light scattering was observed, caused presumably by micellization. Upon further increasing the temperature by 2–3°C the scattering intensity increased

dramatically and the solution became opalescent, most probably because of the formation of a solid precipitate. The process of micellization/precipitation was found to be reversible: upon cooling, the solutions became transparent again. Table 1 summarizes the results for CyA in pure water and 164 mM NaCl. Inspection of Table 1 reveals that the solubility of the cyclosporines increases with decreasing temperature. A plot of $\ln c_{sol}$ versus $1/T$ (van't Hoff plot) yields a straight line, and enthalpies of solubilization of $\Delta H^0_{sol} = -16.4$ kcal/mol ($R = 0.98$) and -12.2 kcal/mol ($R = 0.95$) were found for CyA in water and 0.164 NaCl, respectively.

The solubility of CyA in water has been investigated before.[22,23] Qualitatively, the two studies also reveal a decreased solubility at higher temperatures. Quantitatively, the ultracentrifugation method yields smaller solubilities than the light scattering approach employed here.

The dissolution of CyA-OH in aqueous media is kinetically hindered, and no reproducible results were obtained by simply stirring CyA with H_2O. CyA-OH were therefore prepared as described above, i.e., CyA-OH was dissolved in 1 mL ethanol and the ethanolic solution was stirred slowly into 1 L of cold water (4°C). The saturation limit was determined again by increasing the temperature and recording the temperature of light scattering onset (cf. Table 1). CyA-OH shows an about threefold better solubility than CyA as can be expected on the basis of the additional hydroxyl group. The solubility decreases with temperature and the average enthalpy of solution is $\Delta H = -21.3$ kcal/mol ($R = 0.98$) in the temperature interval of 18.5 to 35°C.

Aggregation of CyA and CyA-OH must occur before the onset of micellization/precipitation but is difficult to detect with conventional spectroscopic means. Using UV and CD spectroscopy a linear increase in the optical density and ellipticity was observed at room temperature even beyond the critical micellar concentration of ~ 30 μM. For the analysis of titration calorimetry experiments nonaggregated solutions were preferred. Thus, all measurements were carried out at cyclosporin concentrations ≤ 15 μM.

Circular Dichroism Spectroscopy of Cyclosporines in Solution and Bound to Lipid Bilayers

The conformations of CyA in single crystals and in apolar solvents were determined by X-ray crystallography and 2D NMR spectroscopy, respectively, and were found to be rather similar. The basic features in solution are intramolecular NH···O=C hydrogen bonds leading to a twisted β-sheet and a type II′β-turn at the amino acids 3 and 4.[24–27]

CD spectra were measured for CyA in pure water and bound to small unilamellar vesicles composed of POPC (Figure 2). The dominant feature of these spectra is a negative CD band centered at $\lambda = 226$ nm, which is characteristic for a β-turn structure (Figure 2A).[28] A mean residue ellipticity of $\Theta = -2.4 \pm 0.04 \cdot 10^4$ deg cm^2 d mol^{-1} (at $\lambda = 226$ nm) was observed for CyA in pure

Table 1. Solubility of CyA and CyA-OH

Compound	Solvent	c (mg/L)[a]	c (μM)	Temperature (°C)[b]
CyA	H_2O	9.29	7.7	38.5
		19.57	16.3	31
		28.59	23.8	24
		106.1	88.2	13
CyA	164 mM NaCl	8.9	7.4	34.5
		13.25	11	31
		17	14.1	27
		22.2	18.5	21.5
CyA-OH	H_2O	24.9	19.7	35
		48.2	38.1	31
		68	53.9	27
		88	69.7	26.5
		104.8	83.0	23.5
		143.3	113.5	21.5
		185	146.5	18.5

[a]Peptide dissolved in 1 mL ethanol and added to 1 L of water at 4°C.
[b]The clear solutions were warmed from 4°C starting temperature in 1°C steps. The temperature at the onset of light scattering was taken as the limit of solubility.

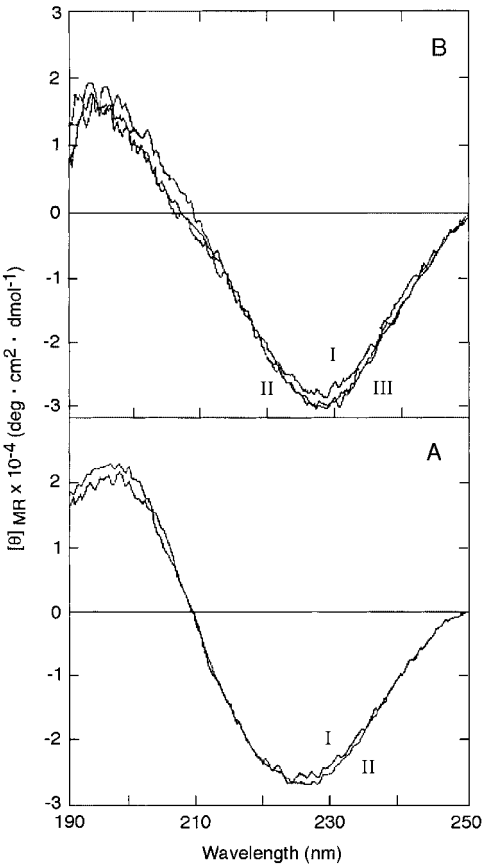


Figure 2. Circular dichroism spectra of CyA. (A) CyA in water. I: $C_{peptide} = 33.8$ μM; II: 15.5 μM; (B) CyA bound to POPC vesicles, I: lipid:CyA ratio 32.3, II: lipid:CyA: ratio 64.5, III: lipid:CyA ratio 128.7; $C_{peptide} = 17.1$ μM.

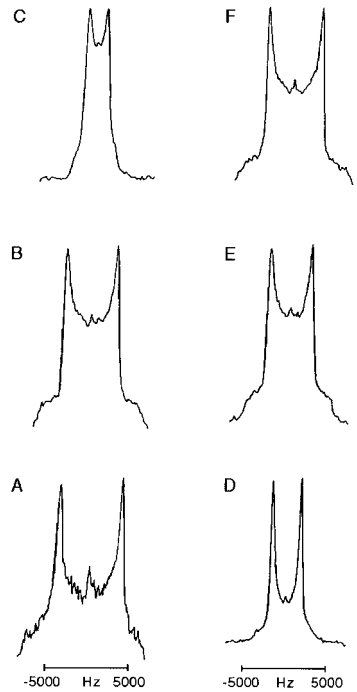
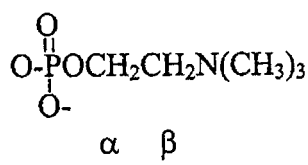


Figure 3. Deuterium NMR spectra of pure POPC membranes (B, E), and of POPC membranes with CyA-NH_3^+ (C, F) or CyA-SO_4^- (A, D). POPC was selectively deuterated at the α-segment (A, B, C) and the β-segment (D, E, F). The amount of bound molecule was $X_b = 0.07$ in A, $X_b = 0.12$ in C, and $X_b = 0.11$ in D and F.



water at room temperature. Interaction of CyA with lipid membranes revealed no significant change in the shape of the CD signal (Figure 2B). The mean residue ellipticity of membrane-bound CyA increased to $\Theta = -3.0 \pm 0.04 \cdot 10^4$ deg cm^2 d mol^{-1} at a lipid-to-peptide ratio of ~130.

A simulation of the CD spectra was based on a standard set for α-helix, β-sheet, β-turn, and random coil CD spectra. Binding of CyA to the lipid membrane increased the β-sheet content from 48 to 53%.

Deuterium NMR Studies of the Phospholipid Headgroup Studies

To simplify the discussion the following notation is used for methylene segments of the phosphocholine head group.

The quadrupole splitting of the α (β) segment is denoted $\Delta\nu_\alpha$ ($\Delta\nu_\beta$). Figure 3 compares ^{2}H-NMR spectra of pure POPC membranes with those containing either CyA-NH_3^+ or CyA-SO_4^-. All deuterium NMR spectra show a single quadrupole splitting, indicating a time-averaged headgroup conformation and a high mobility of the cyclosporines in the lipid membrane, affecting all headgroups during the time of the measurement. The residence time of the peptides at an individual lipid segment is shorter than 10^{-5} s.

The uncharged CyA has almost no effect on the choline head groups. The variation of the headgroup splittings with the amount of bound peptide (mol fraction $X_b = n_{bound\ peptide}/n_{unbound\ peptide}$) is shown in Figure 4A. Compared to previous measurements with other neutral molecules such as cholesterol.[29] CyA is apparently not seen by the phosphocholine polar group.

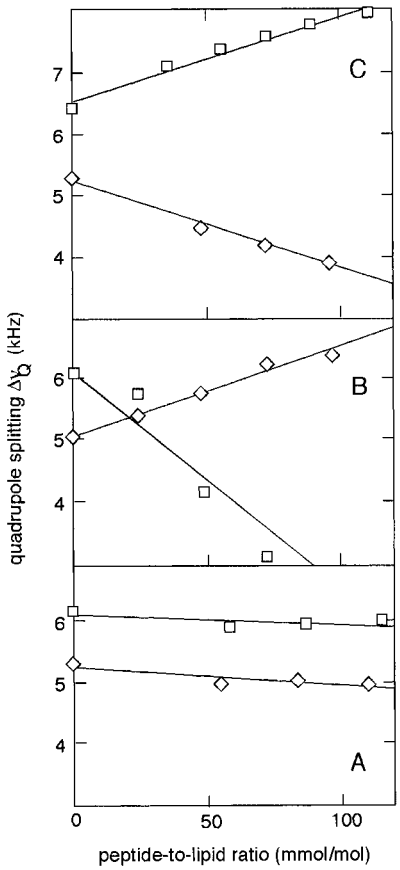


Figure 4. Variation of the quadrupole splittings of α- and β-deuterated POPC with different cyclosporines. (A) CyA, (B) CyA-NH_3^+, (C) CyA-SO_4^-. □: α-deuterated POPC, ◇: β-deuterated POPC.

In contrast, the charged CyA derivatives induce quite large variations in the ^{2}H-NMR spectra of the POPC head group. The adsorption of the positively charged CyA-amine to POPC membranes leads to a counterdirectional change of the two quadrupole splittings, i.e., $\Delta\nu_\alpha$ *de*creases and $\Delta\nu_\beta$ *in*creases (Figure 3C and F). The variation of the quadrupole splittings with amount of bound peptide is summarized in Figure 4A. Linear regression analysis yields $\Delta\nu_\alpha = 6.13 - 33.4\,X_B$ (kHz) and $\Delta\nu_\beta = 5.1 + 15.1\,X_B$ (kHz) with the ratio of the slopes being $m_\alpha/m_\beta = -0.45$. The *inverse response* is obtained for the negatively charged CyA-sulfate (cf. Figure 3A and D). Here, $\Delta\nu_\alpha$ *in*creases while $\Delta\nu_\beta$ *de*creases. Linear regression analysis of the data shown in Figure 4B yields $\Delta\nu_\alpha = 6.42 + 15.2\,X_B$ and $\Delta\nu_\beta = 5.3 - 17.7\,X_B$ with a ratio of the slopes of $m_\alpha/m_\beta = -1.16$.

These results are in agreement with the known response of the phosphocholine head group upon addition of positively or negatively charged species to the membrane surface. In the absence of electric surface charge, the average orientation of the phosphocholine dipole is parallel to the membrane surface as deduced from ^{2}H-NMR [30] and neutron diffraction studies.[31,32] Upon addition of positive surface charges the choline moiety moves towards the water phase, with negative charges a movement towards the membrane interior is induced.[33–35] They are caused by a change in the orientation of the $^-$P-N$^+$ dipole that depends on the sign and the extent of the membrane surface charge.

Deuterium NMR Studies of the Hydrocarbon Chains

The interaction of cyclosporines with the hydrophobic part of the bilayer was investigated with bilayers composed of 1-palmitoyl-2-[9′,10′-$^2H_2$] oleoyl-*sn*-glycero-3-phosphocholine ([9′,10′-$^2H_2$] POPC). The two deuterons at the *cis* double bond give rise to two separate quadrupole splittings of about 13.0 and 2.0 kHz for the C-9′ and C-10′ deuterons, respectively. The origin of this effect is a tilting of the *cis* double bond with respect to the bilayer normal, i.e., the two deuterons attached at the rigid *cis* double bond make a different angle with the axis of motional averaging.[16] Incorporation of CyA, CyA-NH_3^+, or CyA-SO_4^- into the lipid bilayer produces virtually identical changes in the deuterium spectra. The C-9′ quadrupole splitting increases by 0.5–1.0 kHz, the C-10′ splitting decreases by the same amount. At the highest cyclosporin concentrations investigated (~10 mol %), the quadrupole splitting of the C-10′ deuteron is no longer resolvable. Compared to cholesterol,[29] the perturbation of the hydrophobic membrane interior by CyA is again very small, indicating a perfect match between the fluid-like hydrocarbon chains and the peptide surface. No essential differences are observed between cyclosporin fully immersed in the bilayer or anchored by a polar group at the lipid–water interface.

Isothermal Titration Calorimetry (ITC) of Cyclosporin–Membrane Interaction

The reaction enthalpy and the partition constant of the various cyclosporines were measured with high-sensitivity titration calorimetry by injecting POPC phospholipid vesicles into the respective peptide solution (for a review of the method see ref. 20).

The partitioning of the cyclosporines into the membrane produces an endothermic heat

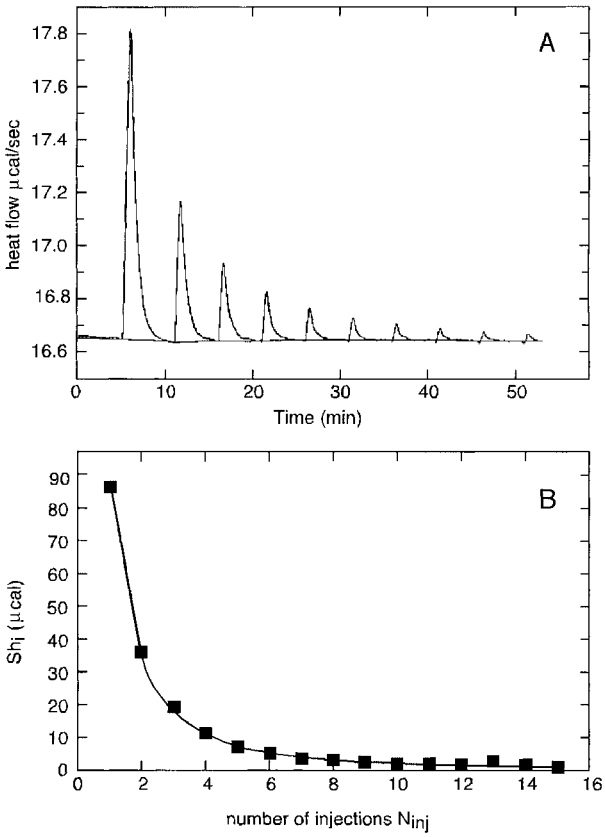


Figure 5. Isothermal titration calorimetry of CyA (13.81 µM) with POPC liposomes (100-nm diameter, $C_{\text{Lipid}} = 34.8$ mM). The calorimeter cell contained the CyA solution and 10 µL injections of the unilamellar vesicle suspension were made at 6-min intervals. (A) heat flow, (B) integrated heat data. Temperature: 28°C.

effect. Figure 5 shows a typical lipid-into-peptide titration with injections of 10 µL aliquots of a 34.8-mM POPC liposome suspension (d ≈ 100 nm) into a 13.81-µM CyA solution. The heat of reaction decreases with increasing number of injections because less and less cyclosporin is available for binding. Control measurements were made by injecting the lipid vesicle suspension into the calorimeter cell filled with pure water only. All heat values were corrected for the heat of dilution. The incorporation of the uncharged cyclosporines into the neutral POPC bilayer can be described by a simple partition equilibrium according to

$$X_b = K C_{p,f} = C_p^0 \cdot \frac{K C_L^0}{1 + K C_L^0} \qquad (1)$$

where $X_b = C_{p,\text{bound}}/C_L^0$ is the degree of binding and $C_{p,\text{bound}}$ is the concentration of peptide bound to lipid of total concentration $C_L^0 \cdot C_p^0$. and $C_{p,f}$ are the total and the free peptide concentration,

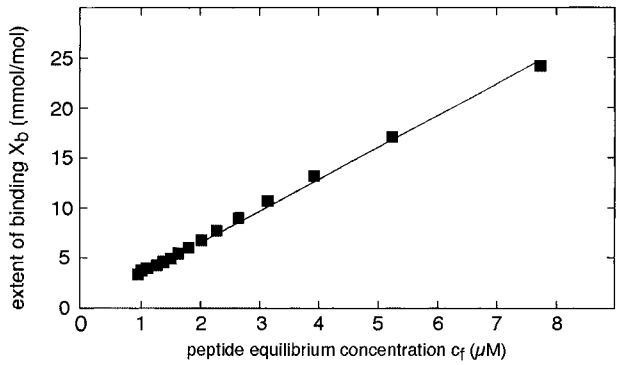


Figure 6. The data of Figure 5 were used to derive the degree of CyA binding, X_b, and the peptide concentration free in solution, C_f. X_b is defined as the molar amount of bound peptide per mole total lipid. A linear relationship is obtained between X_b and C_f with the slope of $K = 5200\ \text{M}^{-1}$.

respectively, and K is the partition constant. Figure 6 shows the linear correlation between the degree of binding, X_b, and the bulk equilibrium concentration c_{eq}. The slope of the solid line represents the binding constant $K = X_b/c_{\text{eq}} = 3200\ \text{M}^{-1}$. The analysis is based on the total lipid and assumes a rapid translocation of the noncharged CyA across the lipid bilayer.

For CyA, an average heat of reaction of $\Delta H^0 = +8.7 \pm 1.2$ kcal/mol and a partition coefficient of $K \approx 4300 \pm 600\ \text{M}^{-1}$ were obtained for 30-nm vesicles (cf. Table 2). The free energy of binding was calculated according to $\Delta G^0 = -RT \ln 55.5$ K and was found to be $\Delta G^0 = -7.4$ kcal/mol. The factor 55.5 accounts for the cratic contribution to the binding.[36,37] Analogous experiments were performed for other vesicle sizes and are summarized in Table 2. Increasing the vesicle size leads to a small decrease in the partition constant.

The binding of CyA-OH to sonicated POPC vesicles is characterized by a smaller partition constant of $K \approx 1500 \pm 200\ \text{M}^{-1}$ reflecting the more polar character of this molecule and its better solubility in water. The binding enthalpy was $\Delta H^0 = 11.5 \pm 0.6$ kcal/mol (Table 2).

Cholesterol exerts a stiffening effect on the fatty acyl chains of the membrane thus increasing membrane ordering.[38,39] Lipid-into-peptide titrations using liposomes containing 30% cholesterol yielded a binding constant for CyA of about one-third of the value found for pure POPC vesicle membranes ($K \approx 1050\ \text{M}^{-1}$). This can be explained by a hindered penetration into the cholesterol-containing membrane due to restricted flexibility of the tightly packed hydrocarbon

Table 2. Partitioning of Cyclosporines Into POPC Lipid Membranes: Thermodynamic Parameters at 28°C

Compound	Vesicle Diameter (nm)	ΔH^0 (kcal/mol)	K^a (M^{-1})
CyA	30	8.7 ± 1.2 ($n = 7$)	4300 ± 600
	200	10.3 ($n = 1$)	3200
	400	7.8 ± 0.9 ($n = 3$)	3200
	30[e]	7.4 ± 0.3 ($n = 2$)	1050
CyA-OH	30	10.2	2000
	100	10.1	800
CyA-NH_3^+	30	8.7 ± 0.5 ($n = 2$)[b,d]	3100 ± 100[f]
CyA-SO_4^-	30	~ 0	3000[c,d,f]

[a]The partition constant was evaluated according to eq. (1). The noncharged cyclosporines were assumed to translocate rapidly from the outer to the inner lipid half-layer and the total lipid, C_L^0, was used to evaluate K_p.

[b]Due to its charged amino group, CyA-NH_3^+ remains on the outer vesicle halflayer. Only 60% (30-nm vesicles) or 50% ($d \geq 100$ nm) of the total lipid is available for CyA-NH_3^+ binding.

[c]Binding constant determined with a POPC monolayer at 32 mN/m (cf. text).

[d]For the charged cyclosporines eq. (1) only provides an approximate description of the binding isotherm because electrostatic repulsions come into play as the first charged molecules bind to the neutral POPC membrane. These can be treated using the Gouy-Chapman theory, leading to a somewhat higher intrinsic binding constant (cf. text).

[e]Membrane composition POPC/cholesterol (70/30 mol %/mol %).

[f]Electrostatic effects not considered.

chains. The measured heat of reaction was $\Delta H^0 = +7.4 \pm 0.3$ kcal/mol.

We have also measured the partitioning of two charged CyA derivatives into POPC bilayers. Because of their charge, the two peptides, CyA-NH_3^+ and CyA-SO_4^-, can only bind to the outer monolayer of lipid vesicles and cannot translocate easily. In evaluating the ITC measurements, all calculations were based on 60% (30-nm vesicles) or 50% (100- and 400-nm vesicles) of the total lipid.

CyA-NH_3^+ binds to 30-nm vesicles with an enthalpy of $\Delta H^0 = 8.7 \pm 1.0$ kcal/mol and an apparent partition constant of $K \sim 3100\ M^{-1}$, i.e., with very similar parameters as the parent compound. However, a closer inspection of the binding isotherm reveals that it does not increase linearly with the peptide concentration. At higher peptide concentration the measured data deviate from the straight line towards smaller binding. This can be explained by electrostatic repulsion counteracting hydrophobic insertion. The binding of the first CyA-NH_3 molecules to the membrane creates a positively charged outer surface which makes the binding of further molecules more difficult. To properly evaluate the binding isotherm, electrostatic effects at the membrane surface were corrected using the Gouy-Chapman theory.[40] In addition, the quantitative evaluation of the binding isotherms of the charged CyA-derivatives was based on a half-sided binding to the outer monolayer only. The binding isotherms are best described with an electric charge of $z_{pep} \approx 0.8$ (pK = 8.2 of amino group) and an intrinsic binding constant of $K \approx 5000\ M^{-1}$.

The calorimetric study of CyA-sulfate showed a complete different picture. No heat signal was obtained after injection of lipid vesicles (30- or 400-nm diameter) to CyA-sulfate solutions. Hence, CyA-SO_4^- either does not bind to lipid membranes or ΔH^0 is accidentally zero. Monolayer penetration experiments were therefore performed in a Langmuir trough. A POPC monolayer was formed at a pressure of 32 mN/m, i.e., the bilayer–monolayer equivalence pressure.[41] CyA-SO_4^- was injected into the subphase, and the area change of the monolayer was monitored at constant pressure. A significant increase in the surface area was observed providing direct experimental proof for the insertion of CyA-SO_4^- into the lipid membrane. Following established methods, [41] an apparent binding constant of $K \approx 3000\ M^{-1}$ was derived, using a surface area requirement of CyA-SO_4^- of 220 Å. It can be concluded that CyA-SO_4^- binds to the POPC membrane with a similar affinity as the other CyA compounds but with a reaction enthalpy which is close to zero.

Binding of Cyclosporines to BSA

Binding studies of cyclosporines to BSA were performed in 10 mM Tris buffer at pH 7.4. The temperature of the microcalorimetry reaction cell

was cooled to 12°C to increase the solubility of CyA. The concentration of CyA was 20.6 μg/mL, BSA was titrated from a stock solution containing 4.7 mg/mL BSA. In several titrations with slightly varying conditions no heat signal was observed. The heat of binding is thus either accidentally zero or no binding occurs.

DISCUSSION

The nature of the interaction of CyA with membrane lipids is controversial. Based on a broadening of the lipid phase transition in multilayer model membranes most authors agree on a location of CyA in the *membrane interior*.[6,9,13] However, it was also concluded that CyA associated with phosphatidylcholine liposomes is localized within the *aqueous compartment* of the liposomes.[42] Both an ordering[9,43] and a disordering[6] of the lipid molecules has been postulated. Finally, different values are found in the literature about the membrane/water partition coefficient of CyA.[4,44] Using deuterium nuclear magnetic resonance and isothermal titration calorimetry we have employed two new methods showing that CyA is localized in the liposomal bilayer and to determine precisely the partition coefficient of CyA in lipid membranes.

Location of CyA and Charged Derivatives in POPC Membranes

The influence of the cyclosporines on the hydrophobic part of the lipid phase was investigated with membranes composed of POPC, which was selectively deuterated at the *cis* double bond, [9′,10′-$^2H_2$]-POPC. Addition of cyclosporines reduced the quadrupole splittings, providing evidence for a more random motion of the lipid chains. The increase in segmental fluctuations was, however, small, and almost identical for all CyA derivatives investigated. Even though an appreciable amount of CyA (up to 10 mol %) was dissolved in the membrane interior, the hydrocarbon chains adjusted to this disturbance with a minimum conformational change. This has been noted before for integral membrane proteins such as cytochrome oxidase[15,45] or sarcoplasmic ATPase[46] as well as for model peptides[47] or even crystal-like proteins.[48] This can be explained by the uneven surface and the flexibility of proteins leading to a "fluid-like" match between the protein outer surface and the surrounding lipid.[49] NMR studies and molecular dynamics calculation have demonstrated that CyA adopts various conformations, suggesting a limited flexibility of this molecule also in the lipid membrane.[50] For the noncharged CyA the membrane perturbation is also propagated towards the head group region. Both the α- and the β-quadrupole splitting of the phosphocholine moiety show a small decrease upon addition of CyA (Figure 4A), indicating a more random movement of the head group.

The charged analogs CyA-NH_3^+ and CyA-SO_4^- are also easily incorporated into the POPC membrane and disorder the hydrocarbon chains to a similar extent as the neutral parent compound. On the other hand, their effect on the phosphocholine head group is quite different. Both compounds lead to distinct changes in the quadrupole splittings of the α- and β-segment. CyA-NH_3^+ decreases the α-splitting and increases the β-splitting, CyA-SO_4^- has exactly the opposite effect. The variation in the quadrupole splittings is by a factor of 10–30 larger than that induced by neutral CyA. The counterdirectional change with $\Delta\nu_\alpha$ decreasing and $\Delta\nu_\beta$ increasing (or vice versa), excludes a more random motion of the lipid headgroup because this would reduce both quadrupole splittings simultaneously.[30] Instead, the observed changes can only be explained by a reorientation of the phospholipid head groups.

Indeed, phospholipid head groups behave as sensors for the electric charge at the membrane surface ("molecular electrometer" model).[33] Addition of positively charged molecules to the lipid membrane moves the N^+ end of the $^-$P-N^+ dipole towards the water phase, whereas negative charges at the membrane surface move it towards the bilayer interior. The chemical nature of the charges is of secondary importance, and similar effects are caused by metal ions, hydrophobic ions, local anesthetics, voltage-sensitive dyes, charged lipids, or peptides.[34] Previous studies have also shown that the ratio of the slopes m_β/m_α is about -0.5 for cationic molecules and about -1.0 for negatively charged molecules. The results obtained for CyA-NH_3^+ and CyA-SO_4^- are completely consistent with these earlier findings, both with respect to the nature and the extent of the observed changes. It should also be noted, that CyA-SO_4^- is the first *negatively charged peptide*, which was investigated by this method. It can be concluded from the ^{2}H-NMR data that the bulk of the CyA molecule is embedded in the bilayer interior, but that the charged residues are positioned in close vicinity to the lipid $^-$P-N^+ dipoles.

Thermodynamics of the Cyclosporin–Membrane Equilibrium

Isothermal titration calorimetry is a general method to measure chemical reactions or physical adsorption processes that are accompanied by the consumption or release of heat. Heat changes are indeed observed for the adsorption/penetration of cyclosporines into lipid membranes. With the exception of CyA-SO_4^-, all peptides bind to the lipid membrane with *endothermic* reaction enthalpies of about 5–10 kcal/mol. A negative free energy, ΔG^0, can hence only arise by a sufficiently large positive entropy change such that $T\Delta S^0$ more than compensates ΔH^0. CyA binding to the lipid membrane thus follows the pattern of the classical hydrophobic effect, which is an entropy-driven phenomenon.[37] However, compared to earlier calorimetric studies on peptide–lipid interactions this result is remarkable for two reasons. First, almost all other peptides investigated so far showed a distinctly exothermic ($\Delta H^0 \ll 0$) heat of binding. Secondly, in all earlier studies ΔH^0 was found to be strongly dependent on the size of the vesicles and changed from exothermic for 30-nm vesicles to endothermic for 400-nm vesicles.[51] In the present studies, the vesicle diameter has almost no influence on the measured ΔH.

A possible explanation for the unusual results obtained with CyA and its analogs could be the remarkably large solvation enthalpy of CyA and CyA-OH. The solubility of CyA and CyA-OH increases with decreasing temperature and the exothermic solvation enthalpy is between -10 and -20 kcal/mol at 30°C. Upon entering the lipid membrane, CyA will loose most of its hydration shell, which is only possible at the expense of a large positive ΔH^0. The gain in van der Waals interaction of the hydrophobic part of CyA upon insertion into the lipid membrane is not large enough to compensate the hydration enthalpy and the residual enthalpy remains positive. For CyA-SO_4^- the two enthalpies may accidentally balance each other, leading to a zero net enthalpy.

Titration calorimetry also allows the measurement of the binding isotherm. The quantitative interpretation of the binding isotherm leads to the elucidation of the binding mechanism and the binding constant. For CyA and its hydroxylated derivative CyA-OH the analysis of the data is straightforward (cf. ref. 20). The binding of the neutral drugs can be described by a simple partition equilibrium according to $X_b = K_p \cdot C_f$. The molar amount of peptide bound per mole lipid, $X_b = n_{\text{bound peptide}}/n_{\text{unbound peptide}}$, is found to be a linear function of the free peptide concentration (C_f). For CyA and CyA-OH the partition coefficients are 4300 M^{-1} and 1500 M^{-1}, respectively, at 28°C and 30-nm vesicles (Table 2). Previously, an ultracentrifugation assay has been used to determine the partition coefficient of CyA and CyA-OH between liposomal membranes and buffer yielding $P = 4000 \pm 500$ for CyA and $P = 500 \pm 60$, respectively,[44] which is in broad agreement with the ITC data. The partition coefficient $P = C_{\text{memb}}/C_f$[44] is numerically comparable to the present partition coefficient $K = X_b / C_f$ because 1 mol lipid has a volume of roughly 1 L and the *mol fraction*, X_b, hence corresponds numerically to the *concentration*, C_{memb}, of drug in the membrane. The octanol/water partition coefficient of CyA was also measured and was found to be 850.[50] This result is consistent with the present membrane partitioning studies, because octanol is a more polar solvent than the lipid membrane. Despite its apparent high lipophilicity, CyA can transfer easily from the liposomal membrane to other membranes, as was shown in other studies.[12] Obviously because of its structure, CyA does not fit perfectly into the membrane environment.

A reduced membrane partition coefficient is obtained if cholesterol is added to the membrane ($K = 1050$ M^{-1}). Cholesterol leads to a stiffening of the fatty acyl chains, making it more difficult to accommodate guest molecules. This reduced capacity for CyA-uptake was also noted for cholesterol containing monolayers.[8]

For the charged molecules CyA-NH_3^+ and CyA-SO_4^- the binding isotherm must be corrected for electrostatic effects using the Gouy-Chapman theory and the bulk concentration C_f must be replaced by the peptide concentration C_M encountered at the membrane surface in solution, immediately above the plane of binding (cf. Ref. 40). With this proviso, the binding of the charged peptides again follows a simple partition equilibrium ($X_b = K_p\, C_M$).

CONCLUDING REMARKS

The present NMR studies provide a detailed picture of the influence of CyA on the ordering of the hydrocarbon chains and the lipid head group. CyA can be incorporated into the membrane up to relatively high concentrations of about 10 mol % without perturbing the membrane. Nevertheless,

a lipid load of at least 2.5 g per day would be necessary to inject the formulated daily CyA dose, which could cause metabolic problems. The measurement of the membrane water partition equilibrium of CyA is hampered by the low water solubility of CyA but is, nevertheless, possible with high-sensitivity isothermal titration calorimetry. In the concentration range investigated, the CyA binding increases linearly with the aqueous concentration of the drug. The binding reaction is endothermic with $\Delta H \sim +10$ kcal/mol and the driving force for the binding reaction is the entropy.

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