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The role of molecular conformation in ion capture by carboxylic ionophores: a circular dichroism study of narasin A in single-phase solvents and liposomes

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Conformational and thermodynamic aspects of cation binding by the carboxylic ionophore narasin A were studied by circular dichroism (CD). In single-phase solvents, dramatic increases in the maximum differential absorption ($\Delta\epsilon$) of the C-11 carbonyl were observed upon the binding of K^+, Na^+ and protons to the free anionic form. These changes were associated with major shifts in the conformation equilibrium between extended and pseudocyclic conformers of narasin. Similar CD changes observed upon the binding of K^+ to narasin A in dimyristoylphosphatidylcholine vesicles provided evidence that in the membrane environment, comparable conformation changes were associated with ion binding. Variation of the polar and protic properties of single-phase solvents was also found to influence the $\Delta\epsilon$ of the cation bound species of narasin A, supporting previous evidence for polarity-mediated modulation of conformation. Comparison of cation binding affinities indicated that in both single-phase solvents and liposomes, narasin had a marked equilibrium selectivity for K^+ over Na^+.

Introduction

The conformations of cation-bound carboxylic ionophores in the solid state and in single-phase solvents have been the subject of extensive studies that have been reviewed recently [1–5]. Implicit in such studies is the assumption that an analogy exists between the solid and isotropic solution states and the ordered anisotropic environment of a phospholipid membrane. Possible limits to such an analogy have been discussed previously [6]. During its cation transport cycle, a carboxylic ionophore will experience a wide range of chemical environments with the cation-binding reaction occurring at the asymmetric and polar aqueous-membrane interface. Fluorescence studies have provided information about the location, kinetics and thermodynamics of carboxylic ionophore interactions with membranes [7–11]. Little is known, however, about how phospholipid membranes modulate carboxylic ionophore conformations and whether major conformational changes occur upon cation binding. In the present study a conformationally sensitive probe, circular dichroism (CD), is used to investigate the cation-binding reactions of the carboxylic ionophore narasin A (Fig. 1) both in single-phase solvents and in phospholipid vesicles. Although CD had been applied profitably

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Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

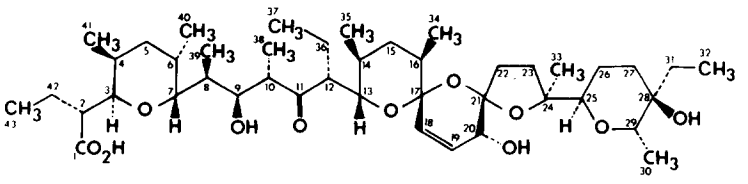

Fig. 1. Structure of narasin A. This compound, which has recently been marketed as an anticoccidal drug [12], functions as an electrically silent exchange diffusion carrier of monovalent cations across cellular membranes [13].

in membrane studies of the channel-forming peptide ionophore gramacidin A [14,15] and the neutral, macrocyclic ionophores valinomycin and enniatin B [16], no such applications to carboxylic ionophores in membranes have been reported.

Materials and Methods

Reagents. All solvents were freshly distilled and shown by flame emission photometry (FEP) to have less than 0.1 μM Na^+ and K^+. Narasin A, the gift of Dr. Robert Hamill of Eli Lilly and Co., was acidified and recrystallized according to the method of Berg and Hamill [17].

Flame emission photometry indicated that the acid contained less than 0.1% of the Na^+ and K^+ salt forms of narasin. Tri-*n*-butylamine (TBA) was distilled under vacuum into plastic and determined by flame emission photometry to have less than 0.1 μM Na^+ and K^+. Tetra-*n*-butylammonium hydroxide (TNBAH) was used without further purification (1 M solution in methanol from Aldrich) and was found to contain 40 mM Na^+ and negligible K^+. The level of Na^+ contamination in the tetra-*n*-butylammonium hydroxide was considered in the analysis of the binding studies in which it was used. The sodium, potassium and ammonium salts used in titrations were dried overnight in vacuo at 100°C before weighing. Flame emission photometry indicated that the Na^+ and K^+ salts each contained less than 0.00013 equivalents of the other.

Dimyristoylphosphatidylcholine (DMPC, Sigma) was shown by flame emission photometry to contain less than 0.005% Na^+ and K^+ by weight. The buffer (Buffer 1) in which DMPC was dissolved for the vesicle preparation (a modification of that used by Kauffman et al. [11]) contained 33 mM tetraethylammonium chloride (Sigma), 5 mM dimethylglutaric acid (Sigma), 5 mM Hepes (Sigma) and 5 mM Ches (BDH), and the pH was adjusted to 8.2 with tetraethylammonium hydroxide (20% aqueous, Sigma). Tetraethylammonium chloride was recrystallized twice from acetonitrile/acetone before use. Buffer 1 was found by flame emission photometry to initially contain 80 μM Na^+ and 4 μM K^+, this was taken into consideration in the analysis of the results. No CD-active components were detected in Buffer 1 in the 250–350 nm range.

Cation-binding studies in single-phase solvents. Narasin acid was dissolved in the designated solvent at a concentration (10–200 μM) close enough to the apparent K_d of the given cation to allow accurate determination of both bound and free cation concentrations during the titrations. Deprotonation of narasin to yield the free anion was performed by the addition of appropriate dilutions of pure tri-*n*-butylamine or 1 M tetra-*n*-butylammonium hydroxide [18–20]. Although the tri-*n*-butylammonium cation may ion pair with the terminal carboxyl anion of narasin in less polar solvents [19], both this cation and the tetra-*n*-butylammonium ion are too bulky, and the positive charge too shielded from polar ligands to be chelated in a tight multidentate complex with narasin. The latter assumption was supported by the fact that narasin, deprotonated in deuterated methanol by tetra-*n*-butylammonium deuteride, has a nuclear magnetic resonance spectrum virtually identical to narasin deprotonated by lithium deuteride and quite distinct from the NMR spectra of the acid and cation-bound forms of narasin [21]. The most obvious explanation for this is that the tetra-*n*-butylammonium cation was too large and Li^+ too small to be chelated with high affinity by narasin.

Binding studies were performed by adding sequential aliquots of KSCN, NaSCN, KCl or NaCl to deprotonated narasin until total saturation of the ionophore was achieved. The proportions of cation-bound versus cation-free narasin present after each addition of cation was determined by monitoring the circular dichroism

spectrum of the solutions with a JASCO J41C spectropolarimeter. The differential absorption ($\Delta\epsilon$) of the peak (negative maximum between 290 and 294 nm) due to the $n \rightarrow \pi^*$ transition of the ketonic carbonyl was found to be sensitive to conformation and cation binding in related carboxylic ionophores [18–20,22,23]. Deprotonation of narasin acid by addition of tri-*n*-butylamine or tetra-*n*-butylammonium hydroxide resulted in a decrease in $\Delta\epsilon_{max}$ and was considered complete when the addition of more base resulted in no further decrease in $\Delta\epsilon_{max}$. Titration of deprotonated narasin with the above-mentioned salts resulted in a saturable increase in $\Delta\epsilon_{max}$. Assuming that the minimum $\Delta\epsilon_{max}$ ($\Delta\epsilon_d$) and maximum $\Delta\epsilon_{max}$ ($\Delta\epsilon_b$) obtained represented the fully dissociated and fully cation-bound forms of the ionophore, respectively, the fractional saturation (ν) of solutions with $\Delta\epsilon_x$ were calculated using Eqn. 1

$$\nu = (\Delta\epsilon_x - \Delta\epsilon_d)/(\Delta\epsilon_b - \Delta\epsilon_d) \quad (1)$$

The binding reaction between the dissociated ionophore and a monovalent cation is first order with respect to each reactant [1,6–8]. Assuming a 1 : 1 ratio of narasin to cation, the free cation concentration (L) with respect to the total analytical concentrations of cation ($[\text{cation}]_{total}$) and narasin ($[\text{narasin}]_{total}$) was calculated using Eqn. 2

$$L = [\text{cation}]_{total} - \nu[\text{narasin}]_{total} \quad (2)$$

Least-squares analyses of plots of ν/L versus ν (Scatchard plots) were used to obtain the slopes and apparent K_d values for Na^+ and K^+.

DMPC vesicles. Small unilamellar DMPC vesicles were prepared by a combination of the procedures of Huang and Thompson [24] and Kauffman et al. [11]. 95 mg of dry DMPC was suspended in 3 ml of Buffer 1, dispersed by vortexing at 35°C and then sonicated under an H_2O-saturated N_2 atmosphere for 2 h at 30°C using an MSE probe tuned to maximum power output. The sonicate was diluted to 8 ml with Buffer 1 and centrifuged at $100000 \times g$ for 60 min to remove large aggregates and titanium flakes from the probe. The Sepharose 4B column chromatography reported in the original procedures was not performed, as it had been reported that this step might cause the generation of more large vesicles than it removed [25]. This vesicle preparation was diluted to a nominal concentration of 3 mM DMPC with Buffer 1 and used within 24 h. The vesicles were placed in an 0.5 cm pathlength CD cell at 20° and 2 μl concentrated narasin acid in ethanol was added to give final concentrations of 50 μM narasin. This phospholipid to ionophore ratio (60 : 1) is well below that necessary to saturate the vesicles. The addition of less than 0.1% ethanol does not perturb the DMPC vesicles [26]. Owing to the high lipophilicity of narasin, it was assumed that the ionophore was partitioned predominantly into the less polar vesicle membrane as has been shown to be the case for other carboxylic ionophores [10,27]. Aliquots of 3–5 M or solid KCl or NaCl were added sequentially to the cuvette up to a final concentration of at least 400 mM. Scatchard analysis of the KCl titration was performed as has been described for the organic solvent titrations.

Results

Deprotonation

Since narasin transports protons as well as alkali cations [13], it was necessary to generate the free anion prior to titration with other cations in order to eliminate complications due to proton competition for the cation-binding site. Deprotonation was performed in ethanol, methanol and 20% aqueous methanol, three solvents which are thought to bracket the polarity range at the aqueous membrane interface where the ion complexation reaction occurs [1].

The deprotonation of narasin acid in methanol with tri-*n*-butylamine, for example, resulted in a 78% decrease in $\Delta\epsilon_{max}$ (Fig. 2). A similar spectrum was obtained for the tetra-*n*-butylammonium hydroxide-deprotonated narasin; however, since tetra-*n*-butylammonium hydroxide is a stronger base than tri-*n*-butylamine, only 1–2 equivalents of tetra-*n*-butylammonium hydroxide were required for deprotonation. Deprotonation caused similar effects on $\Delta\epsilon$ of narasin in ethanol and in 20% aqueous methanol, except that in the latter a lower percentage decrease in $\Delta\epsilon_{max}$ (52%) was observed (Table I). Backtitration of deprotonated narasin with HCl regenerated a CD spectrum identical to the original narasin acid spectrum, indicating that the deprotonation procedure did not irreversibly

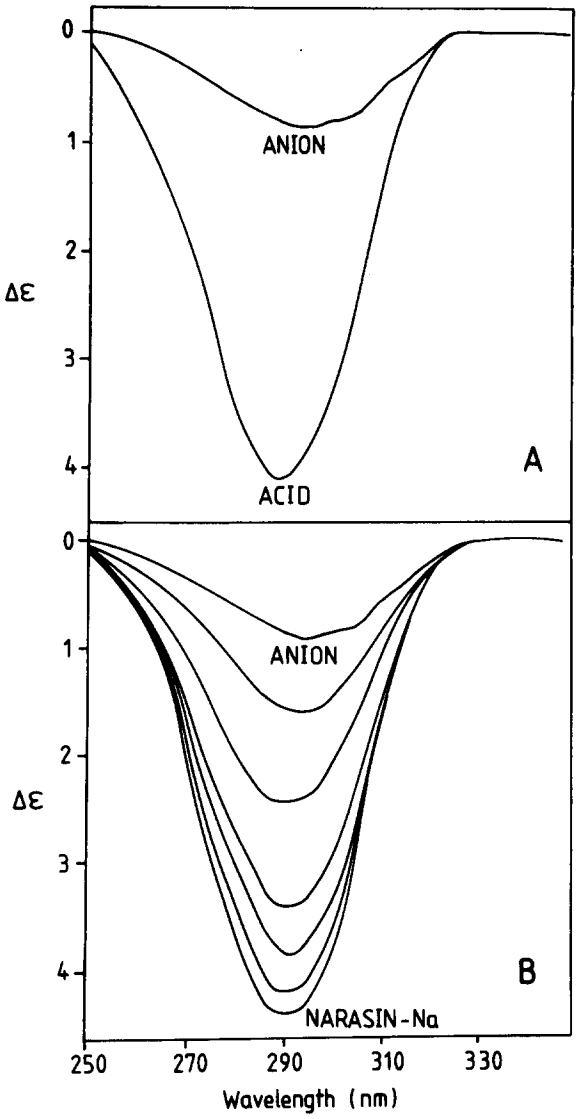

Fig. 2. CD spectra of 200 μM narasin A in methanol (path length = 1 cm). (A) The acid and the tetra-*n*-butylammonium hydroxide-generated anion. This spectrum of the acid is similar to the one previously published [36]. (B) The effect of adding increasing amounts of NaSCN in methanol to tri-*n*-butylamine-deprotonated narasin A. The bottom spectrum represents fully saturated narasin-Na. The terms for $\Delta\epsilon$ are 10^{-3} $cm^2 \cdot mol^{-1}$.

alter narasin. Further evidence that no degradation of narasin A acid in deuterated methanol occurs under these deprotonation conditions has been provided by nuclear magnetic resonance spectroscopy [21].

Cation-binding studies in single-phase solvents

Saturation of deprotonated narasin in single phase solvents with Na^+ or K^+ resulted in an increase in $|\Delta\epsilon_{max}|$ to a magnitude similar to that of the narasin acid spectrum. The effect of adding NaSCN to narasin in methanol is shown in Fig. 2. The Na^+ and K^+ titrations in the three solvent systems yielded linear Scatchard plots and the apparent K_d values summarized in Table II. Contrary to results reported for lasalocid A [19,20], the choice of base for the deprotonation of narasin acid made no significant difference in the apparent K_d values. In 20% aqueous methanol, where both the thiocyanate and chloride salts of the cations could be used as titrants, no significant counter anion-dependent changes in the apparent K_d values were observed.

The peak $\Delta\epsilon_{max}$ values of the anion and cation-bound forms of narasin in a range of solvents of varying polar and protic properties (expressed as Reichard's E_T value [28]) are summarized in Table I. Treatment of these values with the Lorentz correction factors for the varying solvent refractive index did not significantly alter the relative values of $\Delta\epsilon$ [29]. Except for the anomalously high $\Delta\epsilon_{max}$ values obtained for narasin acid and anion in 20% aqueous methanol, the $\Delta\epsilon_{max}$ values of the acid, anion and Na^+-bound form increased with decreasing E_T (i.e., decreasing polar and protic character) of the solvent. The $\Delta\epsilon_{max}$ of the K^+-bound form was insensitive to changes in solvent E_T in the range of 20% aqueous methanol to ethanol.

K^+ binding to narasin in DMPC vesicles

The effect of adding KCl to narasin in DMPC vesicles is shown in Fig. 3. The addition of narasin (50 μM final concentration) caused a modest increase in the $\Delta\epsilon$ (Fig. 3a) to a net value ($\Delta\epsilon_{(b)} - \Delta\epsilon_{(a)}$) of approximately 1 (Fig. 3b) which is similar to that seen previously for the anion in methanol. The latter fact suggested that narasin was mostly, if not completely, deprotonated under those conditions; however, since pK_a values for narasin have not been determined for these or any other conditions, this cannot be said with certainty.

Addition of KCl caused a marked increase in $\Delta\epsilon$ (Fig. 3c–f). The similarity in sign, peak frequency, $\Delta\epsilon_{max}$, and overall profile of the spectra

TABLE I

MAXIMUM $\Delta\epsilon$ VALUES OF NARASIN

Values adjusted for differences in solvent refractive index by the Lorentz correction factors are given in parentheses.

Solvent	E_T	Maximum $\Delta\epsilon$ values of narasin [a]			
		Acid	Anion	Na^+-bound	K^+-bound
n-Hexane	30.9	1.95(1.50)			
Chloroform	39.1	2.05(1.52)			
Ethanol	51.9	1.52(1.19)	0.55(0.43)	1.77(1.38)	1.77(1.38)
Methanol	55.5	1.39(1.11)	0.31(0.25)	1.50(1.20)	1.83(1.46)
20% aqueous methanol		1.55	0.81	1.42	1.84

[a] Units for $\Delta\epsilon$ are 10^{-3} cm^2·mol^{-1} with λ_{max} from 290 to 294 nm.

to those seen for narasin upon cation binding in single-phase solvents provided strong evidence that the observed CD spectral changes were due to narasin and not to some component of the vesicle preparation itself. This was confirmed by the fact that the addition of similar concentrations of KCl to DMPC vesicles alone produced no effect on the CD. Furthermore, it was shown recently that differential light scattering and absorption flattening optical effects are minimal in the CD spectra of small unilamellar DMPC vesicles prepared by sonication [30]. To test for the possibility that an unequal distribution of narasin and/or K^+ across the membrane influenced the magnitude of $\Delta\epsilon$ under apparently K^+-saturated conditions, 10 μM valinomycin was added to break down any existing K^+ gradient; however, no effect on the CD spectrum was detected. Analysis of the binding data by the method described previously yielded in a linear Scatchard plot and an apparent K_d of 8 mM for K^+ (Fig. 4).

TABLE II

APPARENT K_d VALUES OF NARASIN IN SINGLE-PHASE SOLVENTS

Titrant	Ethanol	Methanol	20% Aqueous methanol
NaSCN	1 μM	230 μM	3.1 mM
NaCl	[a]	[a]	2.8 mM
KSCN	[b]	3 μM	93 μM
KCl	[a]	[a]	86 μM

[a] The poor solubility of the titrant in these solvents prevented the determination of these values.

[b] This K_d was too low to be accurately determined by this method.

Addition of up to 400 mM NaCl to an identical preparation of narasin in DMPC vesicles produced no detectable change in the CD spectrum from that seen in Fig. 3b. When KCl was added to

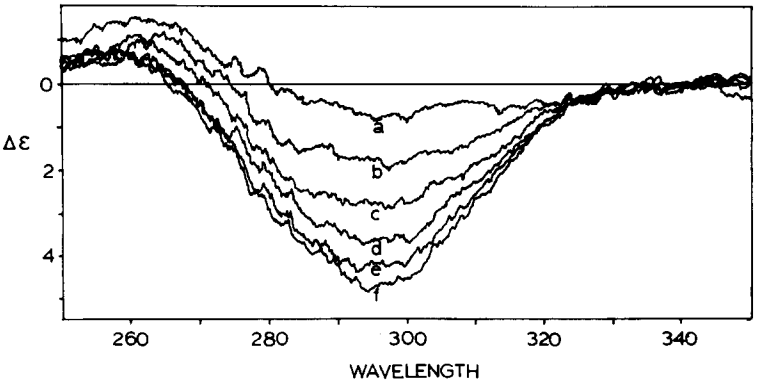

Fig. 3. CD spectra of 50 μM narasin A in DMPC vesicles (3 mM nominal [DMPC]) in Buffer 1, pH 8.2 (cell path length = 0.5 cm). (a) DMPC vesicles alone in Buffer 1. This spectrum is superimposable on that of the H_2O blank and thus represents the instrumental baseline (which was accentuated by the high sensitivity setting required) rather than intrinsic circular dichroic activity of the liposomes themselves. (b) DMPC vesicles + 50 μM narasin, the latter added as 2 μl of a concentrated ethanolic solution of the acid form. (c–f) The effect of adding increasing amounts of KCl to the sample described in (b). The bottom spectrum represents K^+-saturated narasin and was obtained for a [KCl] of approx. 100 mM.

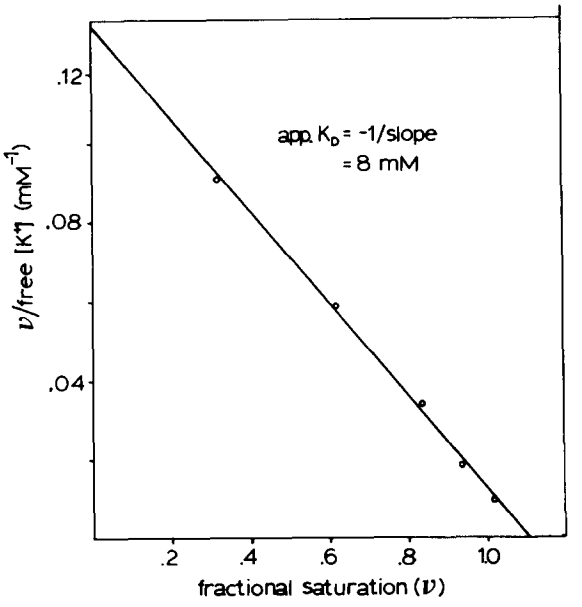

Fig. 4. Scatchard plot of K^+ binding to narasin in DMPC vesicles (cf. Fig. 3). The line drawn represents that to which the data points were best fitted by least-squares analysis.

the same NaCl-laden samples, the usual increase in $\Delta\epsilon$ was observed indicating that nothing was inherently different between vesicle samples used in the KCl and NaCl titrations. This could be explained by one or both of the following: (1) in DMPC vesicles, the apparent K_d of narasin for Na^+ was too high for a detectable proportion of 50 μM narasin to be bound at 400 mM NaCl; (2) the $\Delta\epsilon$ of the Na^+-bound form in DMPC vesicles was much lower than in the single-phase solvents studied, making the difference in $\Delta\epsilon$ between the anionic form of narasin and the Na^+-bound too small to detect with the experimental procedures used. Since in the solution titrations all of the cation inclusion complexes of narasin in a large range of polar and protic environments were characterized by $\Delta\epsilon$ values of at least 4, it seems unlikely that the latter case was dominant. These results are consistent with a marked equilibrium binding selectivity for K^+ over Na^+ in DMPC vesicles.

Discussion

This study has demonstrated that under equilibrium conditions, narasin has a marked binding selectivity for K^+ over Na^+ in liposomes and in single-phase solvents of varying polarities. As expected, increasing the solvent polarity systematically reduced narasin's affinity for cations because of the increased ability of polar solvents to solvate both the cation and the polar ligands of the ionophore in competition with the complexation reaction [19]. The K^+ over Na^+ equilibrium selectivity observed in the liposomes agrees qualitatively with the kinetic transport selectivity previously seen for narasin in erythrocytes [13], and is consistent with the ionophore operating in the equilibrium domain [31]. The fact that the opposite ($Na^+ > K^+$) transport selectivity was observed for narasin in respiring mitochondria [12] suggests that variables other than intrinsic equilibrium binding selectivity of the ionophore can modulate the apparent selectivity observed in complex biological cation transport systems.

It must be emphasized that the apparent binding constants and transport activities of carboxylic ionophores in vesicles may be profoundly influenced by chemical and physical properties of the membrane and the aqueous compartments [6–11,13]. Such factors can vary dramatically between types of cellular membranes. These experiments have indicated that CD is a useful probe for investigating the effects of such factors on the behavior of carboxylic ionophores with appropriate chromophores, such as narasins A and B, salinomycin and lasalocid, in phospholipid vesicles.

The similarity of the CD spectra of the acid, and the K^+ and Na^+ complexes of narasin indicated that the conformations of the three species are similar (Table I). The NH_4^+ complex also gave a comparable spectrum in methanol, with a $\Delta\epsilon$ of $1.78 \cdot 10^{-3}$ $cm^2 \cdot mol^{-1}$. The C-11 carbonyl is located in one of the two conformationally flexible 'hinge' regions of the carbon backbone of the molecule and it has been proposed that its oxygen is involved in chelating cations [8,21,31,32]. Alterations in the differential absorption of its $n \rightarrow \pi^*$ CD band could be attributed to changes in one or a combination of three factors: (1) conformation in the hinge region; (2) solvation of the chromophore [34,35]; and (3) its asymmetric interaction with a bound cation. The fact that the maximum CD value observed did not vary much between complexes with cations of widely different char-

acter (e.g., polarizability) was an indication that the latter factor did not influence the CD spectrum much; however, it was difficult to distinguish the relative contributions of these factors to the spectral changes observed by the CD data alone. It was apparent that the binding of these cations to the narasin A anion induced similar asymmetries in the environment of the chromophore. Our NMR studies of narasin anion and its cation-bound forms in methanol provided evidence that radical backbone torsional rotations occur in the region of the C-11 carbonyl when the ionophore converts from the open non-pseudocyclic conformational state of the free anion to the closed pseudocyclic cation complex [21]. The increase in $\Delta\epsilon$ upon cation binding by narasin in single-phase solvents is likely to be the result of such a conformational change. The similar phenomenon observed in DMPC vesicles provides evidence that the same conformational transformation occurs upon cation binding by narasin in phospholipid membranes.

The general increase in the $\Delta\epsilon_{max}$ values of the acid, anion and Na^+-salt forms of narasin A going from polar to nonpolar solvents was similar to the effects observed with lasalocid [19,20] and salinomycin [18]. As was proposed, these effects were consistent with a solvent E_T-dependent conformational change for these ionophores whereby more cyclic head-to-hydrogen-bonded conformations were induced in solvents with lower tendencies to solvate cations and/or compete with intramolecular hydrogen bonds. The lower E_T sensitivity of the K^+-narasin complex relative to the Na^+ complex can be attributed to its greater stability which is presumably derived from the more optimal dipole-charge interaction of the liganding oxygens with the larger K^+ ion. Because of the conformational rigidity inherent in the tricyclic spirane, the highly substituted A and E rings and the sterically hindered linear-backbone region between ring A and C-10, the liganding cage of oxygens may not be capable of contracting sufficiently around smaller cations such as Na^+ and Li^+ to allow optimal chelation by a ligand oxygens simultaneously. In the latter cases, certain potential liganding groups may preferentially hydrogen bond to polar solvents causing the breaking of the head-to-tail hydrogen bound and the stabilization of more open conformations characterized by reduced $\Delta\epsilon_{max}$ values. Solvents of low E_T value tend to counteract this phenomenon by forcing the conformation into its limiting closed, intra-molecularly hydrogen-bonded form exemplified by the K^+ complex, as is indicated by the approach of the $\Delta\epsilon_{max}$ of the Na^+ complex to those of the K^+ and NH_4^+ complexes as solvent E_T is decreased. We have previously provided evidence from NMR studies that such E_T-dependent conformational changes occur to a limited extent with the free acid form of narasin A [33]. Interpretation of the CD data alone requires caution, however, since it is possible that solvent E_T-induced changes in $\Delta\epsilon$ can be due in part to changes in the solvation of the chromophore.

This study provides the first direct information about the conformational behavior of a carboxylic ionophore in a phospholipid membrane and supports the following model for narasin's in vivo membrane activity. In its cation-free anionic form at the membrane surface, the ionophore assume an extended non-pseudocyclic conformational state that may be stabilized by solvation of the carboxylate and other polar liganding oxygen, and by ion pairing of the carboxylate with the bulky cationic choline moiety of the phospholipid head group. This form of narasin is characterized by a low $\Delta\epsilon$. The conformational equilibrium shifts toward the pseudocyclic complex with high $\Delta\epsilon$ upon cation binding. As the ligand oxygens displace solvent from the cation, major torsional rotations occur in the C10–C13 region of the molecular backbone. The neutral lipophilic complex is then able to diffuse through the membrane interior to the opposing membrane surface where it equilibrates with the cations of the other aqueous compartment.

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