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SHORT COMMUNICATIONS

Equilibrium cation binding selectivity of the carboxylic ionophore narasin A: A comparison with transport selectivities reported in two biological test systems

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Carboxylic ionophores, besides playing increasingly important roles as probes in experimental biology [1], are used extensively as feed additives for the treatment of coccidiosis in poultry and the enhancement of feed efficiency in ruminants [2]. Several ionophores are also potent cardiovascular stimulants with potential therapeutic use in man [3, 4]. Although the precise mechanism by which this diverse range of activities is induced is poorly understood at the cellular level, the molecular basis of activity is the ability of the ionophore to render cell and organelle membranes permeable to specific cations [5].

The cation transport efficiency exhibited by an ionophore in a biological system usually correlates qualitatively with the equilibrium cation selectivity exhibited in one- and two-phase chemical test systems [6]. Transport studies conducted in two biological systems, however, report different ion selectivity ratios for narasin A (Fig. 1), a monovalent cation-selective carboxylic ionophore recently marketed as an anticoccidial drug [2]. In rat liver mitochondria, narasin A selected Na^+ over K^+ [7], whereas the reverse has been reported in a study using human red blood cells [8, 9]. Since it is the ability of narasin A to bind and transport K^+, Na^+ and H^+ that determines its biological activity, establishing the intrinsic cation selectivity ratio is fundamental to understanding the mechanism of action of the drug.

In this study, the alkali ion binding reactions and selectivities of narasin A were investigated in solution by circular dichroism (CD). Factors affecting the solution activities of both narasin A and the alkali cations were considered to ensure that the observed dissociation constants (K_D values) reflect, as closely as possible, the intrinsic binding affinities of the ionophore. In solution, ion binding is a function of the basic complexation chemistry of the ionophore and is uncomplicated by the presence of active metabolic processes that might mask the fundamental ion binding activity of the ionophore. Once the ion selectivity ratio has been established, the effects that metabolic processes may have on observed ionphore selectivity can be better evaluated in mitochondria, erythrocytes and other biological systems.

Materials and methods

Reagents. Narasin A, the gift of Dr. Robert Hamill of Eli Lilly & Co., was acidified and recrystallized according to the method of Berg and Hamill [10] and determined to be pure by proton magnetic resonance [11, 12] and CD [12]. Methanol, ethanol and tri-*n*-butylamine (TBA) were distilled and shown by flame emission photometry to have <0.1 μM Na^+ and K^+ [i.e. concentrations at least 10- to 10^4-fold lower than the apparent dissociation constants measured for these cations (Table 1)]. Tetra-*n*-butylammonium hydroxide (TNBAH) was used as obtained as a 1 M methanolic solution containing <0.1 μM K^+ and 40 mM Na^+. The level of Na^+ contamination in the TNBAH was taken into consideration in the analyses of binding studies in which it was used.

Cation binding studies. Narasin A acid was dissolved in the designated solvent at a concentration (10–200 μM) close enough to the apparent K_D for the given cation to allow accurate determination of both bound and free cation during the cation titrations (see below). To eliminate competition between protons and alkali cations for the binding cavity, narasin A was deprotonated to yield the free anion by addition of appropriate dilutions of TBA or TNBAH [13, 14]. Aliquots of KSCN, NaSCN or NaCl were then added until saturation of the ionophore was achieved. The K_D values were calculated by Scatchard analysis. The proportions of protonated or cation-bound versus free anionic narasin A present after each addition of base or cation were determined by monitoring changes in the CD spectra of the solutions with a JASCO J41C spectropolarimeter. The differential absorption ($\Delta\varepsilon$) of the ketonic carbonyl $n \rightarrow \pi^*$ peaks of narasin A and related ionophores has been found to be sensitive to the ionization and complexation state of the ionophore (Fig. 2) and thereby useful for determining cation K_D values [13–18]. CD is the method of choice, since many carboxylic ionophores lack ionization-sensitive, u.v.-visible absorption spectra at readily accessible wavelengths. If the differential absorption of the fully deprotonated and fully cation-bound forms of the ionophore is denoted as $\Delta\varepsilon_d$ and $\Delta\varepsilon_b$, respectively, the differential absorption of a mixture of the two forms, $\Delta\varepsilon_x$, represents a population weighted average of $\Delta\varepsilon_d$ and $\Delta\varepsilon_b$ according to the equation:

$$\Delta\varepsilon_x = X_d\,\Delta\varepsilon_d + X_b\,\Delta\varepsilon_b$$

where X_d is the mole fraction of free anionic narasin A and X_b is the mole fraction of cation bound narasin A. Consequently, the fractional saturation, v, at each point in the titration with a given alkali cation can be calculated as the ratio of the net and maximum changes in $\Delta\varepsilon$:

$$v = (\Delta\varepsilon_x - \Delta\varepsilon_d)/(\Delta\varepsilon_b - \Delta\varepsilon_d)$$

Since narasin forms 1:1 complexes with cations [9], the free cation concentration (L) was calculated as the difference between the total analytical concentration of cation and the amount bound by narasin A, $v[\text{narasin})_{\text{total}}$. The apparent K_D for the cation was then determined from a plot of v/L versus v (a Scatchard plot).

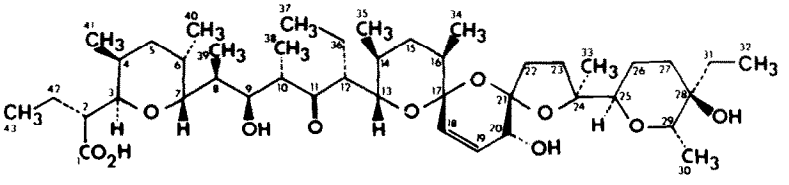

Fig. 1. Structure of narasin A.

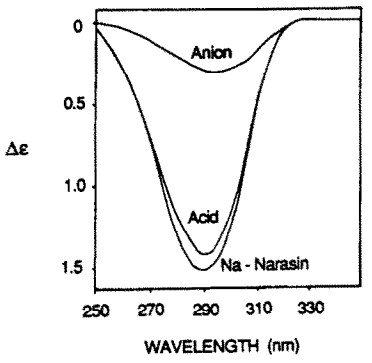

Fig. 2. CD spectra of the acid, the free anion and the Na-bound forms of narasin A in methanol (pathlength = 1 cm; [narasin] = 200 μM). The anionic form was generated from the acid by adding sequential aliquots of TNBAH until no further decrease in $|\Delta\varepsilon|_{max}$ was observed. This required 1.1 equivalents of TNBAH. The Na^+-saturated form was then obtained by the addition of aliquots of a concentrated standardized solution of NaSCN in methanol until no further spectral change was observed. The terms for $\Delta\varepsilon$ are $l \cdot mol^{-1} \cdot cm^{-1}$.

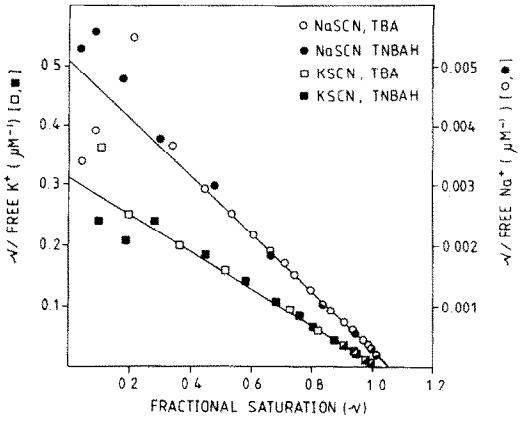

Fig. 3. Scatchard plots of K^+ and Na^+ binding to TBA- and TNBAH-deprotonated narasin A (200 μM) in methanol. The titrations and the calculations of v and L were performed as described in Materials and Methods. The lines drawn represent those to which the data points from the titrations of TBA-deprotonated narasin were best fitted by least squares analyses.

Results and discussion

The narasin A species of prime consideration for ion complexation within membranes is the free anion. The anion combines with a monovalent cation at a reaction plane near the membrane surface to form a 1:1 electrically neutral zwitterionic complex [5, 19, 20]. Two bases, TBA and TNBAH, were used to generate narasin A anion in solution to ascertain if the K_D values were influenced by the nature of the deprotonating agent. Since the tri- and tetra-alkyl ammonium cations produced by deprotonation are too bulky to form multidentate complexes with the ionophore, their abilities to compete with alkali ions for the binding cavity are expected to be minimal [12, 13, 17, 18]. However, in the case of the carboxylic ionophore lasalocid A, TBA and TNBAH differentially influence K_D values by forming ion pairs with the terminal carboxylate [14]. Deprotonation with TBA resulted in a 48–78% decrease in $\Delta\varepsilon_{max}$ depending upon solvent polarity (Table 1). Within experimental error, the same decreases in $\Delta\varepsilon_{max}$ were obtained with TNBAH. Back-titration of narasin A anion in methanol with HCl regenerated a CD spectrum identical to the original narasin A acid spectrum, indicating that the deprotonation procedure did not degrade the ionophore. A more detailed examination of the deprotonation–reprotonation reaction in deuterated methanol by ^{1}H-NMR confirmed that no structural changes had occurred [11, 12].

Titration of the anion with the chloride or thiocyanate salts of Na^+ and K^+ resulted in a saturable increase in $\Delta\varepsilon_{max}$ to a magnitude similar to that of the original narasin acid spectrum (Table 1). The slopes of the Scatchard plots and therefore the calculated K_D values were independent, within experimental error, of whether the anion was generated with TBA or TNBAH (Fig. 3). Steric crowding around the carboxylate of narasin A may preclude the formation of ion pairs with bulky alkyl ammonium ions. The K_D values were also independent of whether the alkali ion was introduced as the Cl^- or SCN^- salt (Fig. 4), indicating that the nature of the counter ion was of little consequence to the cation selectivity under high polarity conditions.

Solvent polarity has an effect on observed K_D values and on ion selectivity ratios. The carboxylic ionophore salinomycin, for example, undergoes an inversion in the K^+:Na^+ selectivity with extreme changes in polarity. Fluorescent and CD studies conducted on ionophores bound to membranes indicate the polarity of the reaction plane where ion complexation occurs to be close to that of methanol [18, 19]. To ensure that the observed K_D values of narasin A are not dramatically affected by slight changes in the polarity of this microenvironment, two additional solvents bracketing the polarity of methanol were used (Table 1). In all three solvents, the equilibrium selectivity of narasin was $K^+ > Na^+$. This suggests that slight differences in polarity at the membrane reaction site that might arise from differences in membrane lipid composition should not significantly affect rank ion selectivity and would not induce an inversion in selectivity.

Table 1. Maximum $|\Delta\varepsilon|$ and apparent K_D values of narasin A in single phase solvents*

Parameter	Ethanol	Methanol	20% Aqueous methanol
Polarity (E_T)†	51.9	55.5	60.5
$\|\Delta\varepsilon\|$ (Acid)	1.52	1.39	1.55
$\|\Delta\varepsilon\|$ (Anion)	0.55	0.31	0.81
$\|\Delta\varepsilon\|$ (Na^+-bound)	1.77	1.50	1.42
$\|\Delta\varepsilon\|$ (K^+-bound)	1.77	1.83	1.84
K_D (Na^+)	1.07 μM ± 0.35	230 μM ± 8.0	3.1 mM ± 0.15
K_D (K^+)	≪1 μM‡	3.47 μM ± 0.43	93 μM ± 2.0

* $\Delta\varepsilon$ is given in units of $l \cdot mol^{-1} \cdot cm^{-1}$. The apparent K_D values represent the mean ± S.E. of a minimum of three independent determinations.

† E_T values are empirically derived measures of solvent polarity [21].

‡ This K_D was too low to be determined accurately by this method.

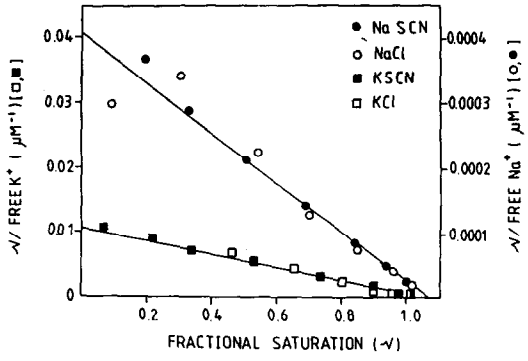

Fig. 4. Scatchard plots of the binding of Na^+ and K^+ to TNBAH-deprotonated narasin A (200 μM) in 20% aqueous methanol. The titrations were performed with solutions of both the chloride and thiocyanate salts of the alkali cations. The parameters v and L were calculated as described in Materials and Methods. The lines drawn represent those to which the data points from the thiocyanate salt titrations were best fitted by least squares analyses.

The K^+:Na^+ selectivity demonstrated by narasin A in erythrocytes qualitatively reflects the equilibrium selectivity seen in each of the three solvents. However, the reported value of 1.65 [8, 9] is considerably attenuated when compared to the equilibrium selectivities calculated from the data in Table 1. To test for the possibility that [Na^+, K^+]-ATPase activity might counter ionophore transport and mask ionophore-induced fluxes, we treated erythrocytes with ouabain and determined the K^+:Na^+ selectivity under conditions previously described [9]. The calculated selectivity of 1.72 is not significantly different from that observed in the absence of ouabain. The ion complexation–decomplexation reactions that occurred at the membrane reaction plane are extremely fast relative to movement of the complexes across the membrane interior [22, 23]. Consequently, the rates of translocation across the membrane are proportional to the relative concentrations of the ion complexes at the reaction plane, which is, in turn, a function of ion selectivity, if the monovalent complexes are assumed to have identical diffusion coefficients [23]. The discrepancy in selectivity ratios calculated from transport and equilibrium experiments may indicate that this assumption is not completely valid.

The cation dependence of respiratory acceleration induced by ionophores in mitochondria is dependent on the nature of the oxidizable substrate. In the case of beauvericin, respiration is promoted most strongly by K^+ when glutamate is the substrate, but by Na^+ when succinate or β-hydroxybutyrate is the substrate [24]. Henderson *et al.* [25] observed a reversal of valinomycin-induced increases in mitochondrial respiration upon the addition of nigericin with all substrates except succinate, β-hydroxybutyrate and proline. These results suggest that the leveling of mitochondrial cation gradients perturbs coupled anion and cation fluxes, producing secondary metabolic inhibitions that can obscure the primary effect of the ionophore. Since both the chemical and less metabolically active erythrocyte test systems report a $K^+ > Na^+$ selectivity for narasin A, the inverted selectivity observed in the mitochondrial study is most likely the result of an active metabolic process superimposed over the ionophore transport activity. So although respiring mitochondria are commonly used to determine rank ion selectivities, the system appears to be complicated by conditional factors.

In summary, circular dichroism was used to study the cation binding reactions of the carboxylic inophore narasin A in solution. The apparent equilibrium dissociation constants for Na^+ and K^+ were calculated in a range of solvents with polarities bracketing that of the microenvironment near the membrane-aqueous interface where *in vivo* complexation reactions are believed to occur. The results indicate that, contrary to the conclusions of a transport study conducted in respiring rat liver mitochondria and consistent with a transport study in human erythrocytes, narasin A has an intrinsic binding selectivity of K^+ over Na^+.

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