Spin-Label Studies on the Origin of the Specificity of Lipid–Protein Interactions in Na^+,K^+-ATPase Membranes from *Squalus acanthias*†

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ABSTRACT: The pH dependence and salt dependence of the lipid–protein interactions of phosphatidic acid, phosphatidylserine, and stearic acid with Na^+,K^+-ATPase membranes from *Squalus acanthias* have been studied with spin-label electron spin resonance spectroscopy, using lipids with nitroxide labels on the 14-position C atom of the *sn*-2 chain. For phosphatidic acid and stearic acid, the fraction of motionally restricted spin-label increases with increasing pH, with pK_a's of 6.6 and 8.0, respectively. In contrast, the pK_a of stearic acid in the bulk lipid environment of the membrane is estimated from spin-label spectroscopy to be ≈6.6. The fraction of motionally restricted phosphatidylserine spin-label remains constant over the pH range 4.7–9.2. In the fully dissociated state the fractions of motionally restricted spin-labeled phosphatidic and stearic acids decrease with increasing salt concentration, reaching an approximately constant value at [NaCl] = 0.5–1.0 M. For stearic acid the net decrease is comparable to that obtained on protonation, but for phosphatidic acid the decrease is considerably smaller (by ~55%) than that obtained on protonating the lipid. The fraction of motionally restricted phosphatidylserine spin-label varies relatively little with salt concentration up to 1 M NaCl. Direct electrostatic effects alone cannot account for the whole of the observed specificity of interaction of the two phospholipids with Na^+,K^+-ATPase membranes.

Lipid–protein interactions are important features of the structure of biological membranes. They determine, at least in part, the way in which the activity of membrane enzymes and transport proteins is controlled by the lipids and how these integral proteins are sealed into the lipid membrane. Electron spin resonance (ESR)[1] spectroscopy of spin-labeled lipids has proved to be of considerable utility in the investigation of such interactions, because of its favorable time scale [for a review, see Marsh & Watts (1982)].

Previous spin-label studies of lipid–protein interactions in Na^+,K^+-ATPase membranes have revealed a motionally restricted lipid population interacting with the membrane protein, which is not present in dispersions of the extracted membrane lipids (Brotherus et al., 1980; Marsh et al., 1982; Esmann et al., 1985). An interesting selectivity of the interaction has been observed with different spin-labeled lipids, the degree of association at pH 7.4 being in the order cardiolipin > phosphatidylserine ≈ stearic acid ≳ phosphatidic acid > phosphatidylglycerol ≈ phosphatidylethanolamine ≈ phosphatidylcholine (Esmann et al., 1985). The greatest specificity is for negatively charged phospholipids, but this does not bear a direct relation to the net charge on the lipid, and in addition the selectivity pattern is different from that observed with some other membrane proteins (Marsh, 1983). This suggests that the selectivity does not have purely a simple electrostatic origin, as was previously deduced from experiments with the single-chain lipids stearyl methyl phosphate and stearyltrimethylammonium methanesulfonate (Brotherus et al., 1980).

In this paper we investigate in more detail the origin of the specificity of lipid–protein interactions with phosphatidic acid, stearic acid, and phosphatidylserine in Na^+,K^+-ATPase membranes from *Squalus acanthias*. Comparison of the results of salt and pH titration suggests that only a relatively small proportion of the selectivity of the negatively charged phospholipids can be screened by salt and hence is of direct electrostatic origin.

Materials and Methods

Na^+,K^+-ATPase-rich membranes were prepared from the rectal gland of *Squalus acanthias* according to the method of Skou & Esmann (1979). The membranes correspond to the "white pellet" enzyme in Table I of the preceding reference. Typical SDS gel scans are given, for example, in Esmann et al. (1979). Specific activity was typically 1100 μmol of $P_i \cdot mg^{-1} \cdot h^{-1}$, which is somewhat lower than that reported in Skou & Esmann (1979), presumably because of less efficient removal of the nonactive proteins by deoxycholate. Na^+,K^+-ATPase ($\alpha_2\beta_2$ dimer) was estimated both from the specific activity and from the phosphorylation level (approximately 2 nmol of $P_i \cdot mg^{-1}$) to comprise approximately 50% of the total membrane protein. Enzyme activity was measured as described in Skou & Esmann (1979) in the presence of albumin and in the absence of deoxycholate. The membranous enzyme was stored in 20 mM histidine and 25% glycerol (pH 7.0) at −20 °C. For the ESR experiments the enzyme was transferred to other buffers by pelleting and resuspending in an excess of the appropriate buffer. Sodium phosphate buffers in which the phosphate concentration was maintained constant at 10 mM were used for the pH titration, and 20 mM Tris, pH 9.2, with the appropriate concentration of NaCl was used for the

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[1] Abbreviations: ESR, electron spin resonance; Na^+,K^+-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 14-PASL, 14-PCSL, and 14-PSSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphoric acid, -phosphocholine, and -phosphoserine; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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salt titration. The pH was adjusted with NaOH or HCl by use of a Radiometer electrode. The enzyme activity is stable over the pH range 5.2–9.2 at low temperatures (~0 °C) in the absence of salt. Activity is lost relatively rapidly outside this pH range, particularly at higher temperatures. Addition of salt increases the enzyme stability. Experiments were performed on several different microsomal preparations with little significant variation between preparations. The enzyme was active after the ESR measurements.

The stearic acid spin-label 14-SASL was synthesized essentially according to Hubbell & McConnell (1971), and the corresponding phosphatidylcholine spin-label 14-PCSL was prepared according to Boss et al. (1975). The phosphatidic acid spin-label 14-PASL and the phosphatidylserine spin-label 14-PSSL were prepared by head-group exchange catalyzed by phospholipase D (Comfurius & Zwaal, 1977). Further details may be found in Marsh & Watts (1982a).

Membranes in 20 mM histidine, 0.1 M NaCl, and 1 mM CDTA, pH 7.4, were spin-labeled at a level of 1–2 mol % relative to membrane lipid from a 1–2 mg/mL solution in ethanol, incubated overnight, washed twice, and introduced into 1 mm diameter capillaries for ESR measurement. Buffers were changed during the washing process to obtain those required for the ESR experiment. ESR spectra were recorded on a Varian E-12 9-GHz spectrometer, and digital data collection and spectral subtraction were performed by using a PDP 11/10 dedicated computer with interactive graphics. Spectral subtraction was performed as previously described (Marsh, 1982; Marsh & Watts, 1982b). Fluid component line shapes for the subtraction were obtained from the extracted membrane lipids dispersed in the appropriate buffer. The motionally restricted component line shape was obtained from 14-PCSL in sonicated vesicles of dimyristoylphosphatidylcholine in the gel phase (at either 0 or 2 °C). The same motionally restricted line shape was used for all subtractions in a given titration series. The maximum error in location of the subtraction end point is estimated to be ±0.03 in the fraction of motionally restricted lipid. In almost all cases the agreement between the complementary subtractions was far better than this. Effective order parameters were calculated from the expression:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \frac{a_0'}{a_0} \quad (1)$$

where $2A_{\parallel}$ is the maximum, outer hyperfine splitting and $A_{\perp}$ is obtained from the minimum, inner hyperfine splitting $2A_{min}$ according to (Griffith & Jost, 1976)

$$A_{\perp}\,(\mathrm{G}) = A_{min}\,(\mathrm{G}) + 1.4[1 - (A_{\parallel} - A_{min})/[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})]] \quad (2)$$

The isotropic hyperfine splitting constant in the lipid is

$$a_0 = \tfrac{1}{3}(A_{\parallel} + 2A_{\perp}) \quad (3)$$

and the corresponding value in the single crystal host in which the principal values of the hyperfine tensor A_{xx}, A_{yy}, and A_{zz} were measured (Jost et al., 1971) is $a_0' = {}^1/_3(A_{xx} + A_{yy} + A_{zz})$. Further details of sample preparation and experimental methods are given in Esmann et al. (1985).

RESULTS

The ESR spectra of the 14-SASL stearic acid label in Na^+,K^+-ATPase membranes from *Squalus acanthias* dispersed in 10 mM phosphate buffer at different pHs are given in Figure 1. The spectra all consist of two separate components, corresponding to environments in which the spin-label has different rotational mobilities. As observed previously (Esmann et al., 1985), one component corresponds to the spectra observed from 14-SASL undergoing anisotropic motion in dispersions of the extracted membrane lipids (data not shown). The other component (which is not found in the spectra of the extracted lipids) is resolved as two peaks in the outer wings of the spectra (indicated by the arrows in Figure 1) and corresponds to spin-labeled lipids whose motion is considerably restricted relative to that in the fluid environment of the extracted lipids. This second component is assigned to the lipid population interacting directly with the integral membrane proteins [cf. Marsh et al. (1982)]. From Figure 1 it can be seen that the proportion of this second, motionally restricted component decreases relative to the fluid lipid component as the pH of the suspending buffer is reduced.

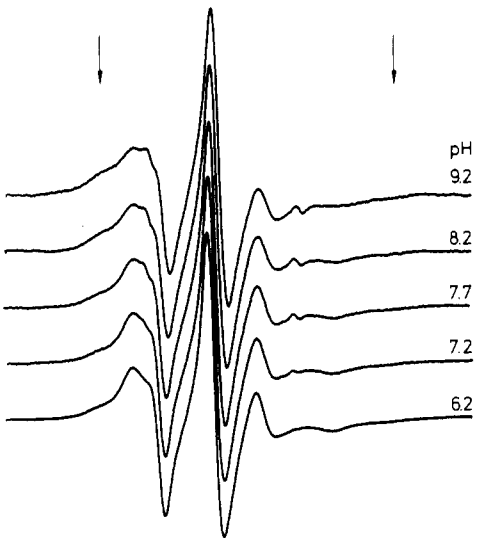

FIGURE 1: ESR spectra of 14-SASL stearic acid spin-label in Na^+,K^+-ATPase membranes in 10 mM Na_2HPO_4 as a function of pH. T = 4 °C; scan width = 100 G. The motionally restricted component, not present in the spectra of the extracted membrane lipids, is indicated by the arrows.

The relative proportions of the motionally restricted and fluid lipid components can be quantitated by subtraction of the digitized spectra, as described previously (Esmann et al., 1985). Consistent values were obtained from complementary subtractions, removing either the fluid component by using a spectrum from the extracted lipids or the motionally restricted component by using a suitable slow-motion spectrum from gel-phase lipids. (It will be noted that there are small differences in the fluid component line shape as a function of pH. Therefore, it is essential to use spectra of the extracted lipids recorded at the same pH and salt concentration for the subtractions.)[2] The fraction f of motionally restricted lipid determined in this way is given as a function of pH in Figure 2. A very clear titration behavior is found that can be fitted to the expression:

$$f = f_{min} + (f_{max} - f_{min})/(1 + [H^+]/K_a) \quad (4)$$

with $f_{min} = 0.21$, $f_{max} = 0.43$, and $pK_a = -\log K_a = 8.00$. Here, f_{min} and f_{max} are the fractions of motionally restricted label in the protonated and dissociated forms, respectively. Sig-

[2] For the motionally restricted component the best match was obtained with a constant line shape. This is to be expected since the spectra are in the slow motion regime and thus are not very sensitive to small perturbations in their dynamic properties.

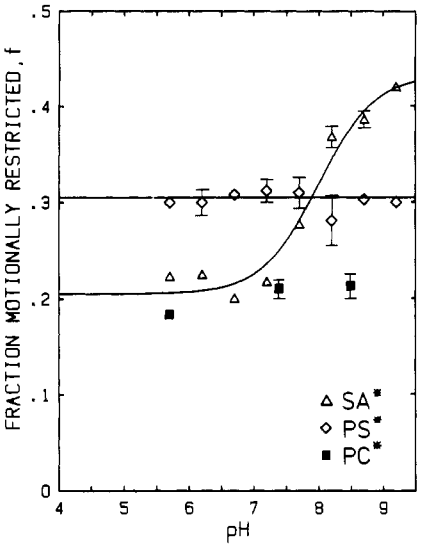

FIGURE 2: pH dependence of the fraction of motionally restricted lipid spin-labels in Na^+,K^+-ATPase membranes in 10 mM sodium phosphate: (△) stearic acid spin-label, 14-SASL; (◇) phosphatidylserine spin-label, 14-PSSL; (■) phosphatidylcholine spin-label, 14-PCSL. The curve for 14-SASL represents a least-squares fit giving a titration between $f_{min} = 0.21$ and $f_{max} = 0.43$ with $pK_a = 8.00$.

nificantly, the fraction of motionally restricted lipid obtained for fully protonated stearic acid is very close to that found for spin-labeled phosphatidylcholine (■ in Figure 2). Also shown in Figure 2 are data obtained in a similar way for the 14-PSSL phosphatidylserine spin-label. In this case, although the protein displays a selectivity for phosphatidylserine, the fraction of motionally restricted spin-label is independent of pH, because the pK_a of phosphatidylserine lies outside the pH range studied.

For comparison, the pK_a of the stearic acid spin-label in the fluid lipid component of the membranes and in the extracted membrane lipids has been determined by titration of the effective order parameter, outer hyperfine splitting, and the isotropic hyperfine splitting constant for the 5-SASL positional isomer [cf. Barratt & Laggner (1974)]. As seen from Figure 3, a pK_a of ca. 6.6 is obtained both in the fluid membrane lipids and in the extracted lipids. This is very close to the values obtained similarly for both the 5-SASL and 14-SASL positional isomers in the model system of dimyristoylphosphatidylcholine + 50 mol % cholesterol: $pK_a \sim 6.6$ and 6.8, respectively (data not shown). The method of obtaining the pK_a from the effective order parameter or hyperfine splittings is somewhat empirical, since at higher temperatures the spectra from 5-SASL in dimyristoylphosphatidylcholine + 50 mol % cholesterol consist of two partially resolved components, indicating that the protonated and unprotonated forms are not in fast exchange on the ESR time scale. Under these circumstances the effective pK_a is probably an upper limit. Two components are not seen in the spectra of 5-SASL in the membranes at higher temperatures, nor is there a measurable titration effect on the spectral splittings of 14-SASL in membranes.

The pH dependence of the spectra of the 14-PASL phosphatidic acid spin-label in Na^+,K^+-ATPase membranes is given in Figure 4. Again the spectra consist of two components, and the proportion of the motionally restricted component decreases with decreasing pH. The salt dependence of the spectra is also given in Figure 5. Here the changes are not as great as with pH titration, but the proportion of motionally restricted lipid decreases somewhat with increasing salt concentration. The pH dependence and salt dependence of the fraction of motionally restricted 14-PASL spin-label determined by spectral subtraction are given in Figure 6. Again the values obtained from the complementary subtractions are in good agreement. The pH titration can be fitted to eq 4 with values $f_{min} = 0.16$, $f_{max} = 0.34$, and $pK_a = 6.59$. In this case the minimum value of the fraction of motionally restricted lipid is apparently somewhat less than that obtained for phosphatidylcholine, although the experimental uncertainty in this region is quite large. On increasing the salt concentration for a sample at high pH, the fraction of motionally restricted 14-PASL spin-label initially falls quite rapidly, then reaching a constant value at approximately 0.4 M NaCl. This limiting

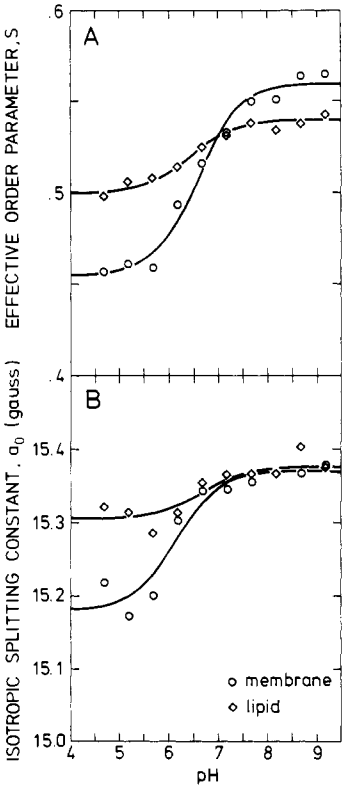

FIGURE 3: (A) Effective order parameter, S, and (B) isotropic hyperfine splitting constant, a_0, as a function of pH for the 5-SASL stearic acid spin-label in membranes (○) and in extracted lipids (◇) from Na^+,K^+-ATPase membranes. $T = 37$ °C. The buffer used was 10 mM sodium phosphate throughout.

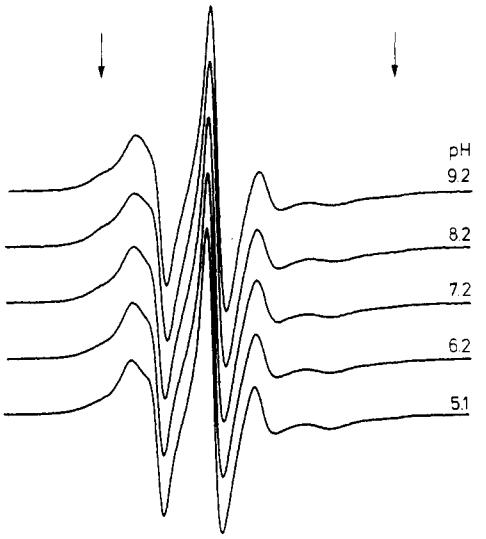

FIGURE 4: ESR spectra of 14-PASL spin-label in Na^+,K^+-ATPase membranes in 10 mM sodium phosphate as a function of pH. $T = 4$ °C; scan width = 100 G.

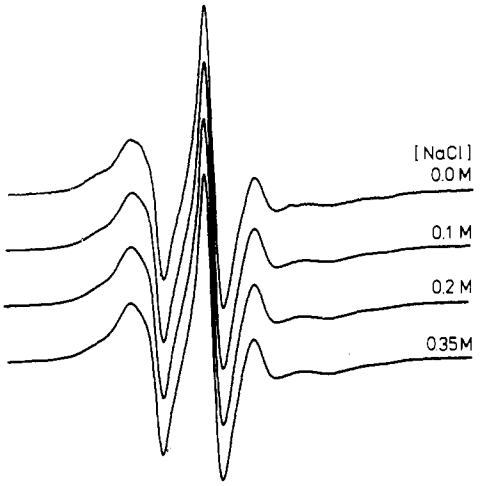

FIGURE 5: ESR spectra of 14-PASL spin-label in Na^+,K^+-ATPase membranes in 20 mM Tris, pH 9.2, as a function of NaCl concentration. T = 4 °C; scan width = 100 G.

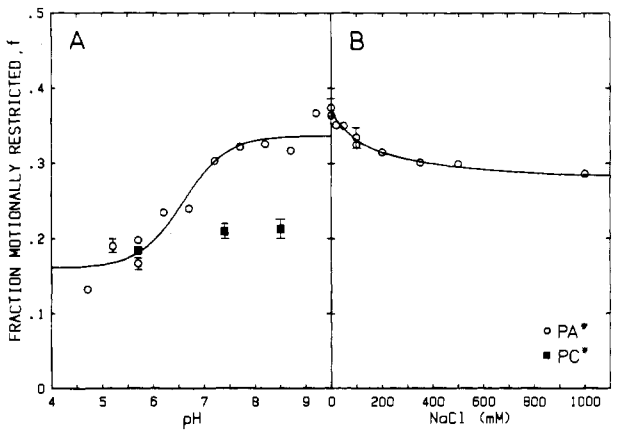

FIGURE 6: (A) pH dependence in 10 mM sodium phosphate and (B) salt dependence in 20 mM Tris, pH 9.2, of the fraction of motionally restricted 14-PASL phosphatidic acid spin-label in Na^+,K^+-ATPase membranes. (■) Fraction of motionally restricted phosphatidylcholine spin-label, 14-PCSL. The curve in panel A represents a least-squares fit giving a titration between f_{min} = 0.16 and f_{max} = 0.34 with pK_a = 6.59.

value obtained in high salt is, however, much greater than the minimum value obtained on titrating to low pH.

The salt dependence of the fraction of motionally restricted 14-SASL stearic acid spin-label and of 14-PSSL phosphatidylserine spin-label is given in Figure 7. The results for stearic acid are somewhat similar to those for phosphatidic acid, except that in this case the salt-induced decrease is considerably larger. At high salt concentrations, [NaCl] ~ 1–2 M, the fraction of motionally restricted 14-SASL is similar to that obtained on titration to pH 5.7 or with the phosphatidylcholine spin-label (cf. Figure 2). In contrast, practically no change in the fraction of motionally restricted 14-PSSL phosphatidylserine spin-label was found with increasing salt concentration.

Discussion

The results of the pH titration of both the phosphatidic acid and stearic acid spin-labels demonstrate that the selectivity of the lipid–protein interaction is strongly dependent on the protonation state of the lipid. A clear titration behavior is obtained with a characteristic effective pK_a of 6.6 for phosphatidic acid and 8.0 for stearic acid. In contrast, no titration is observed for phosphatidylserine, the pK_a of which is expected

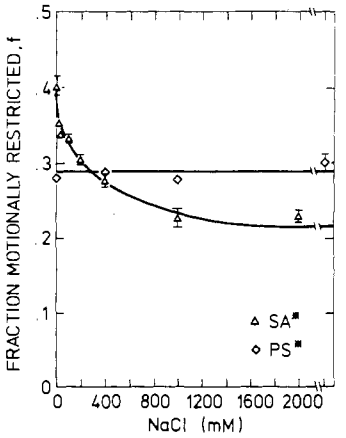

FIGURE 7: Salt dependence of the fraction of motionally restricted lipid spin-label in Na^+,K^+-ATPase membranes in 20 mM Tris, pH 9.0, for the stearic acid spin-label, 14-SASL (△), and in 30 mM histidine, pH 7.6, for the phosphatidylserine spin-label, 14-PSSL (◇). The highest concentration point for 14-PSSL is 3 M NaCl.

to lie below the range of measurement. This latter result is interesting, since it suggests that there are no titratable groups on the protein within the range pH 4.7–9.2 that contribute to the lipid–protein interaction. Presumably, those groups that might contribute positively (e.g., lysine and arginine) and negatively (e.g., glutamic and aspartic acids) to the interaction have pK_a's lying outside the range investigated. Significantly, the effective pK_a of the 14-SASL stearic acid for the lipid–protein interaction (pK_a ~ 8.0) is considerably higher than that determined for the 5-SASL stearic acid isomer either in the fluid lipid environment of the membrane or in dispersions of the extracted membrane lipids (pK_a ~ 6.6). Since little dependence of the effective pK_a on spin-label isomer position was found in the model system dimyristoylphosphatidylcholine + 50 mol % cholesterol, it seems likely that the observed difference corresponds to a true shift in the effective pK_a of the fatty acid at the lipid–protein interface from that in the bulk lipid regions of the membrane. An interesting consequence of this difference in apparent pK_a is that the lipid molecule could take up and release protons on diffusing to and from the protein surface, hence giving rise to localized proton pulses that might potentially be used in transport processes.

The pK_a of a titratable group at the membrane interface, pK_a^i, can differ from that in the bulk aqueous phase, pK_a^0, due to pK_a shifts induced by the electrostatic enhancement of the surface hydrogen ion concentration, ΔpK_a^{el}, and due to the lower polarity at the interface, ΔpK_a^P [see, e.g., Fernández & Fromherz (1977) and Cevc et al. (1981)]:

$$pK_a^i = pK_a^0 + \Delta pK_a^{el} + \Delta pK_a^P \quad (5)$$

For stearic acid in water p$K_a^0 \approx 4.9$. The ΔpK_a^P shift is positive, since the forward association reaction $SA^- + H^+ \rightleftharpoons SAH$ is stabilized by the lower polarity at the interface and has a value of $\Delta pK_a^P \approx 1.1$ (Fernández & Fromherz, 1977). The electrostatic shift depends on the surface charge density σ and can be estimated from diffuse double layer theory [see, e.g., Träuble (1976)] to be $\Delta pK_a^{el} = (2/\ln 10)\sinh^{-1}(\sigma/c)$ with $c = (8000\epsilon\epsilon_0 RTI)^{1/2}$, where I is the ionic strength and ϵ the interfacial dielectric constant. The Na^+,K^+-ATPase membranes contain approximately 5% negatively charged lipids (Esmann et al., 1980); thus, for $I \approx 0.03$ (10 mM sodium phosphate, pH 9.2) and $\epsilon \approx 30$: $\Delta pK_a^{el} \approx 0.8$. Hence, the expected interfacial pK_a^i in the fluid lipid regions of the membrane is pK_a^i ~ 6.8, in quite good agreement with the measured value of 6.6.[3] The difference in the apparent pK_a

of stearic acid between the fluid lipid regions and the lipid–protein interface is less easy to interpret. In principle, the shift could arise either from a lower polarity at the protein interface or from an increased (local) electrostatic enhancement due to additional negative charges on the protein. These factors are difficult to predict, but it seems unlikely that a binding site for negatively charged lipids would have an appreciably enhanced H^+ ion concentration. An effective dielectric constant $\epsilon \sim 10$–20 would be required to give an additional polarity-induced shift of this size (Fernández & Fromherz, 1977), or an increased negative surface charge density of −1 e/5.5 nm^2 would be required to give a comparable electrostatic shift. Comparison with phosphatidic acid is interesting since the situation seems to be exactly the opposite; the effective $pK_a \approx 6.6$ at the lipid–protein interface corresponds very closely to the $pK_a^0 = 6.65$ of glycerol phosphate in water. It would seem that the interfacially induced shifts in the fluid lipid regions of the membrane (which are expected to be similar to those for stearic acid) have been exactly canceled out by the further shifts at the lipid–protein interface. This would argue for somewhat different modes of association or different association sites giving rise to the specificity of interaction of phosphatidic acid and of stearic acid.

Increasing salt concentration causes a screening of the electrostatic interaction between protein and lipid, giving rise to a decreased degree of association (cf. Figures 6 and 7), as is also observed on protonation of the lipid. However, for phosphatidic acid, the screening effect reaches a saturation value at salt concentrations greater than 0.5–1.0 M NaCl, for which the degree of protein–lipid association is still much greater than that for either the protonated lipid or phosphatidylcholine (cf. Figure 6). For phosphatidylserine the situation is more extreme since there is almost no effect of salt on the fraction of motionally restricted lipid. Thus electrostatic effects alone cannot account for the whole of the selectivity of lipid–protein interaction that is observed with these two negatively charged phospholipids. In some respect this bears similarities to the comparative effects of protonation and salt-induced screening on the gel to fluid phase transition of negatively charged lipid bilayers (Cevc et al., 1980). Clearly, the lipid selectivity arises from the detailed chemical nature of the groups involved and not simply their net charge. As with phospholipid bilayer phase transitions (Cevc & Marsh, 1985), the degree of hydration of the phospholipid head groups, and the possible displacement of water of hydration on interaction with the protein, will play an important part.

In contrast, a considerably greater fraction of the motionally restricted stearic acid spin-label can be displaced by salt (Figure 7). The limiting value observed at high salt is only very slightly higher than that found on removing the charge by protonation (cf. Figure 2) and is also comparable to that observed for the zwitterionic phosphatidylcholine. Thus it appears that the selectivity of interaction of stearic acid is essentially of simple electrostatic origin, in common with previous findings on other single-tailed lipids (Brotherus et al., 1980). It will be recalled, however, that in these latter experiments the degree of association of the fully screened, charged lipids was different from that of a similar, single-tailed neutral lipid. Again, this suggests a detailed dependence on the exact chemical structure of the lipid involved.

Table I: Thermodynamic Parameters of Lipid Spin-Label Head-Group Selectivity in Na^+,K^+-ATPase Membranes as a Function of Protonation and Ionic Strength (I)[a]

lipid	I	f	K_r^L/K_r^{PC}	$\Delta G^\circ_L - \Delta G^\circ_{PC}$ (cal/mol)
PA^{*2-}	0.03	0.34	1.9	−360
PA^{*2-}	1.0	0.29	1.5	−240
PA^{*-}	0.03	0.16	0.7	180
SA^{*-}	0.03	0.43	2.8	−570
SA^{*-}	1.0	0.22	1.1	−30
SA*	0.03	0.21	1.0	0
PS^{*-}	0.03	0.30	1.6	−260
PS^{*-}	1.0	0.29	1.5	−240
PC*	0.03	0.21	1.0	0

[a] $T = 4$ °C.

The selectivities in the different protonation states and at different ionic strengths can be compared by using the equation for equilibrium association with the protein (Brotherus et al., 1981; Knowles et al., 1979):

$$(n_f^*/n_b^*) = n_t/(N_1K_r) - 1/K_r \quad (6)$$

where $(n_f^*/n_b^*) = (1-f)/f$ is the ratio of the double-integrated intensities of the fluid and motionally restricted components in the ESR spectrum, n_t is the total lipid/protein ratio in the membrane, N_1 is the number of lipid association sites on the protein, and $K_r \approx [L^*P][L]/[L^*][LP]$ is the average association constant of the spin-labeled lipid relative to the background membrane lipid, defined in terms of moles of lipid. In terms of the true relative association constant K_r^0, defined by activities rather than concentrations:

$$K_r = K_r^0(\gamma_{L^\bullet}\gamma_{LP}/\gamma_{L^\bullet P}\gamma_L) \quad (7)$$

where $\gamma_{L^\bullet}$ and γ_L are the activity coefficients of the spin-labeled and unlabeled lipid, respectively, and $\gamma_{L^\bullet P}$ and γ_{LP} are similarly defined for the lipid–protein complex. In reconstituted systems for which lipid/protein titrations have been performed by varying n_t, it has been found that selectivity arises principally from changes in the average association constant, K_r, rather than in the number of sites, N_1 (Knowles et al., 1981; Brotherus et al., 1981; Brophy et al., 1984). Thus the selectivity relative to a given reference lipid, e.g., 14-PCSL, can be expressed as the ratio of the relative association constants given by $(n_f^*/n_b^*)^{PC}/(n_f^*/n_b^*) = K_r/K_r^{PC}$. These relative association constants and the differential free energies of association, $\Delta G^\circ - \Delta G^\circ_{PC} = -RT \ln (K_r/K_r^{PC})$, are summarized in Table I.[4] Clearly, the pattern of selectivity does not correspond to the net charge on the lipid. This is in agreement with the interpretation of the dependence on ionic strength: that direct electrostatic effects cannot solely account for the observed selectivity. The largest specificity is found for stearic acid in its singly charged form. This is associated even more strongly with the protein than is phosphatidic acid in the doubly charged form, and in the singly charged form the latter has a very low degree of association, comparable to or less than that of phosphatidylcholine. The free energies associated with the various lipid selectivities are quite small, but it must be remembered that these refer to average association constants for all $N_1 \approx 60$ sites on the protein. If the specificity arose from just a few sites, the free energies of

[3] It will be noted that the Fernández & Fromherz (1977) analysis has also been used by Ptak et al. (1980) to interpret interfacial pK shifts of fatty acids in egg phosphatidylcholine bilayers. ESR spectral effects of fatty acid ionization similar to those observed originally by Barratt & Laggner (1974) and to those seen here have also been reported by the same workers (Sanson et al., 1976; Egret-Charlier et al., 1978).

[4] The values for the fraction of motionally restricted lipid differ slightly from those quoted previously (Esmann et al., 1985), due to the different salt and buffer concentrations used and also to differences in the degree of enrichment of the preparations in Na^+,K^+-ATPase.

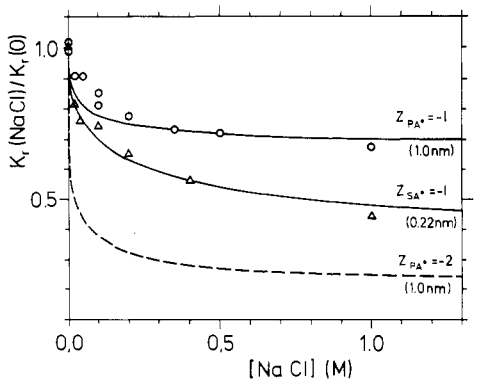

FIGURE 8: Salt-induced screening of the relative association constant of 14-PASL phosphatidic acid (○) and 14-SASL stearic acid (△). Experimental points are deduced from the data of Figures 6B and 7 and are normalized to the value in the absence of salt. Lines are theoretical curves deduced from the Debye–Hückel theory according to eq 9. For stearic acid the solid line is calculated with $Z_{SA^\bullet} = -1$ and $a_{SA^\bullet} = 0.22$ nm. For phosphatidic acid the solid line is calculated with $Z_{PA^\bullet} = -1$ and $a_{PA^\bullet} = 1.0$, and the dashed line is calculated with $Z_{PA^\bullet} = -2$ and $a_{PA^\bullet} = 1.0$ nm.

association would be considerably larger. On the other hand, low free energies could favor fine control of site occupancy if a particular lipid had a regulatory role.

The salt dependence of the selectivity can be understood, at least semiquantitatively, in terms of the Debye–Hückel theory of electrolytes. The effect of increasing ionic strength is to reduce the activity of the charged species involved in the interaction according to [see, e.g., Robinson & Stokes (1955)]

$$\ln \gamma_i = \frac{-Z_i^2 e^2}{8\pi\epsilon_0\epsilon kT}\left(\frac{\kappa}{1 + \kappa a_i}\right) \qquad (8)$$

where Z_i is the charge on species i, a_i is the effective interaction distance of species i with counterions, and the inverse Debye–Hückel screening length is $\kappa = (2000 N_A e^2 I/\epsilon_0\epsilon kT)^{1/2}$, I being the electrolyte ionic strength.[5] The average relative lipid association constant in the presence of ions, K_r, can thus be written in terms of the association constant in the absence of screening, K_r^0, as follows (cf. eq 7): $K_r = K_r^0(\gamma_{L^\bullet}\gamma_P/\gamma_{L^\bullet P})$, where $\gamma_{L^\bullet}$, γ_P, and $\gamma_{L^\bullet P}$ are the activity coefficients of the lipid, protein, and lipid–protein complex, respectively, in the presence of ions. Hence, from eq 8

$$\ln (K_r/K_r^0) = \frac{-e^2\kappa}{8\pi\epsilon_0\epsilon kT}\left[\frac{Z_{L^\bullet}^2}{1 + \kappa a_{L^\bullet}} + \frac{Z_P^2}{1 + \kappa a_P} - \frac{(Z_P + Z_{L^\bullet})^2}{1 + \kappa a_{L^\bullet P}}\right] \qquad (9)$$

The net charge on the protein Z_P is unfortunately not known. If $Z_P \gg Z_{L^\bullet}$, there will be appreciable cancellation of the last two terms in eq 9. As an approximation we have taken a single net excess charge ($Z_P = +1$) and an effective protein radius $a_P = 3.5$ nm. The last term in eq 9 can then be neglected. From eq 6 the ratio of experimental association constants in the presence and absence of salt is given simply by $K_r(NaCl)/K_r(0) = (n_f^*/n_b^*)_0/(n_f^*/n_b^*)_{NaCl}$. These values for phosphatidic acid, 14-PASL, and stearic acid, 14-SASL, are plotted as a function of salt concentration in Figure 8. A good fit of eq 9 to the experimental data for stearic acid can be obtained if one takes $a_{SA^\bullet} = 0.22$ nm and $Z_{SA^\bullet} = -1$, along with the previously mentioned values for the protein. The value of $a_{SA^\bullet}$ required for the fit is not unreasonable in view of the sizes of the ions involved. For phosphatidic acid the situation is different; the fit is not nearly so good, and a rather large value of the effective interaction diameter is required: $a_{PA^\bullet} = 1.0$ nm. In addition, the value taken for the effective charge on the lipid molecule is half that of phosphatidic acid at pH 9.2. As seen from Figure 8, values calculated with $Z_{PA^\bullet} = -2$ decrease much more steeply with increasing salt concentration than do the experimental results. Thus, although the electrostatic contribution to the lipid–protein association with stearic acid is close to that predicted theoretically, the electrostatic effects in the interaction with phosphatidic acid (and phosphatidylserine) are considerably smaller than expected theoretically. This is in line with the fact that in the singly charged state the association of phosphatidic acid is no greater than that for the uncharged phosphatidylcholine.

In summary, the selectivity of lipid–protein interactions in Na^+,K^+-ATPase membranes depends in detail on the chemical nature of the groups involved. Direct electrostatic effects for the two phospholipids studied are relatively weak and contribute to only part of the observed specificities. Lipid hydration and other chemical effects must also be important. The high selectivity observed with the stearic acid label suggests that the lipid chains (single chain vs. double chain) may also make significant contributions. The results of Figure 6 indicate that small changes in pH or ionic strength can cause relatively large changes in the amount of specific lipid associated with the protein, if the local ionic strength is low or if one is in the region of the lipid pK_a. These effects, as well as the difference in pK_a between the bulk lipids and the lipids at the protein interface, may have significant functional effects on the protein activity. It has already been demonstrated that free fatty acids are capable of inhibiting Na^+,K^+-ATPase (Skou, 1964).

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[5] Strictly speaking, the Debye–Hückel theory applies only to very dilute solutions. However, as has long been realized, it is capable of describing the behavior up to quite high electrolyte concentrations, if a_i is treated as an adjustable parameter (Robinson & Stokes, 1955).

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Blood Group A Determinants with Mono- and Difucosyl Type 1 Chain in Human Erythrocyte Membranes†

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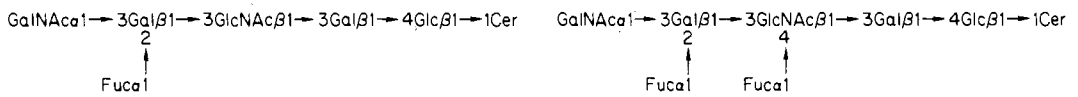
ABSTRACT: Application of a monoclonal antibody defining monofucosyl type 1 chain A (AH21) revealed the presence of a glycolipid having the same thin-layer chromatography mobility as A^a but showing a clear reactivity with AH21. This glycolipid was detectable in Le^{a-b-} erythrocytes but not in Le^{a+b-} or Le^{a-b+} erythrocytes. Another monoclonal antibody defining difucosyl type 1 chain A (HH3) detected the presence of a glycolipid component reacting with this antibody in Le^{a-b+} erythrocytes but not in Le^{a+b-} or Le^{a-b-} erythrocytes. The component defined by monoclonal antibody AH21 and that defined by HH3 were isolated and characterized by ^1H NMR spectrometry and methylation analysis as having the structures



The ^1H NMR spectra of these glycolipid antigens were characterized by resonances for anomeric protons that are identical with those of glycolipids with type 1 chain previously isolated but distinctively different from those of type 2 chain analogues. Resonances reflecting ceramide composition are characteristic for these antigens from human erythrocytes and are distinguishable from those of the same antigen from other sources.

Four types of blood group A glycolipids, A^a, A^b, A^c, and A^d, which differ in the length and branching status of their carrier carbohydrate chains, have been isolated and characterized from human erythrocyte membranes. These components carry the same blood group A determinant (GalNAcα1→3-[Fucα1→2]Gal), and their carrier carbohydrates are mainly of type 2 chain structure (Galβ1→4GlcNAcβ1→3Gal) [see, for review, Hakomori (1981)]. Other major carriers of blood group ABH antigens in human erythrocytes are band 3 and band 4.5 glycoproteins, and the majority of their carbohydrates (lactosaminoglycans) also consist of type 2 chain (Fukuda et al., 1979; Finne, 1980). In addition, A_1 erythrocytes contain a trace quantity of globo-A (GalNAcα1→3[Fucα1→2]-Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer) (Clausen et al., 1984) and a relatively large quantity of the repetitive A determinant (GalNAcα1→3[Fucα1→2]Galβ1→-3GalNAcα1→3[Fucα1→2]Galβ1→4GlcNAcβ1→3Galβ1→-R) (type 3 chain; Clausen et al., 1985), both of which are absent in A_2 erythrocytes. Blood group Lewis antigens (Le^a and Le^b) of erythrocytes are not synthesized in hematopoietic

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