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Spin-Label Studies of Lipid–Protein Interactions in (Na^+,K^+)-ATPase Membranes from Rectal Glands of *Squalus acanthias*

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ABSTRACT: Lipid–protein interactions in (Na^+,K^+)-ATPase-rich membranes from the rectal gland of *Squalus acanthias* have been studied by using spin-labeled lipids in conjunction with electron spin resonance (ESR) spectroscopy. Lipid–protein associations are revealed by the presence of a second component in the ESR spectra of the membranes in addition to a component which corresponds very closely to the ESR spectra obtained from dispersions of the extracted membrane lipids. This second component corresponds to spin-labeled lipids whose motion is very significantly restricted relative to that of the fluid lipids in the membrane or the lipid extract. A stoichiometry of approximately 66 lipids per 265 000-dalton protein is found for the motionally restricted component of those spin-labeled lipids (e.g., phosphatidylcholine) which show least specificity for the protein. This corresponds approximately to the number of lipids which may be accommodated within the first shell around the $\alpha_2\beta_2$ protein dimer. A selectivity of the various spin-labeled lipids for the motionally restricted component associated with the protein is found in the following order: cardiolipin > phosphatidylserine ≈ stearic acid ≳ phosphatidic acid > phosphatidylglycerol ≈ phosphatidylcholine ≈ phosphatidylethanolamine ≈ androstanol.

(Na^+,K^+)-ATPase (EC 3.6.3.1) is a transport enzyme which pumps sodium antiport to potassium coupled to ATP hydrolysis. A highly enriched membranous preparation is obtainable from the rectal gland of *Squalus acanthias* (Skou & Esmann, 1979) in which the ATPase is present as an integral membrane protein associated with much of its endogenous lipid. The active form of the enzyme is an $\alpha_2\beta_2$ dimer of molecular weight 265 000 (Esmann et al., 1980), similar to findings on the enzyme obtained from other sources. A topic of considerable interest is the interaction of the (Na^+,K^+)-ATPase with its membrane lipid environment.

Electron spin resonance (ESR)[1] spin-label studies have shown that large integral membrane proteins are capable of appreciably restricting the mobility of the lipid chains in contact with the hydrophobic surface of the protein [for a review, see Marsh & Watts (1982)]. On the one hand, a charge selectivity has been demonstrated in the interaction of single-chain spin-labels with (Na^+,K^+)-ATPase preparations from *Electrophorus electricus* (Brotherus et al., 1980). On the other hand, a head-group selectivity has been found in the interaction of spin-labeled phospholipids with cytochrome oxidase (Knowles et al., 1981) and several other integral membrane proteins (Marsh, 1985). In the present study, we investigate the interaction of a series of different spin-labeled phospholipids with the (Na^+,K^+)-ATPase from *Squalus acanthias*. A marked selectivity is observed for various phospholipid head groups, indicating a specific interaction with certain phospholipids, namely, phosphatidylserine, phosphatidic acid, and cardiolipin. From measurements of the nonspecific interactions, it is possible to estimate the total number of lipid molecules in contact with the intramembranous surface of the (Na^+,K^+)-ATPase.

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) but omitting treatment with saponin. Typically, the (Na^+,K^+)-ATPase constituted about 70% of the total membrane protein, and the specific activity was 1500 μmol of P_i mg^{-1} h^{-1}. The preparation contained 5–6 mol of residual deoxycholate per mol of enzyme (1 mol of deox-

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[1] Abbreviations: ESR, electron spin resonance; *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; *n*-PASL, *n*-PESL, *n*-PCSL, *n*-PSSL, and *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphoric acid, -phosphoethanolamine, -phosphocholine, -phosphoserine, and -phosphoglycerol, respectively; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycero-3-phospho]-*sn*-glycerol; ASL, 17β-hydroxy-4′,4′-dimethylspiro[15α-androstane-3,2′-oxazolidin]-3′-yloxy; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMPC, dimyristoylphosphatidylcholine.

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ycholate per 100 mol phospholipid + cholesterol). Membrane lipids were extracted with $CHCl_3/CH_3OH$ (2:1 v/v). The buffer used was 20 mM histidine, 100 mM NaCl, and 1 mM CDTA, pH 7.4. Inorganic phosphate was determined according to Bartlett (1959) and cholesterol by the cholesterol oxidase assay (Boehringer Mannheim, West Germany). The phospholipid content of the preparation was 0.87 mg of phospholipid/mg of protein, and the cholesterol content was 0.29 mg of cholesterol/mg of protein.

The stearic acid spin-labels with the nitroxide group on the C-14 or C-16 position, 14-SASL and 16-SASL, were synthesized essentially according to the method of Hubbell & McConnell (1971). The corresponding phosphatidylcholine spin-labels, 14-PCSL and 16-PCSL, were prepared according to the method of Boss et al. (1975). Phospholipid spin-labels with other head groups were prepared from the phosphatidylcholine spin-label and the corresponding alcohol by base exchange, using phospholipase D (Comfurius & Zwaal, 1977; Watts et al., 1979). The cardiolipin spin-label 14-CLSL was made essentially according to Cable et al. (1978) with modifications as described in Knowles et al. (1981). The androstanol spin-label ASL was obtained from Syva, Palo Alto, CA.

The spin-labels were introduced into the membrane suspension (4 mg of protein in 5 mL of buffer) at a level of 1–2 mol % relative to membrane lipid, either as a small volume of concentrated solution in ethanol or as a dried film on the internal surface of the tube. (Similar results were obtained with both methods.) After incubation overnight at room temperature, excess label (and ethanol) was removed by centrifugation and washing. Control experiments showed that ethanol at the concentrations used ($\leq$1 % v/v), or the added spin-labels, had no effect on enzyme activity. Lipid dispersions were labeled by mixing ca. 1 mol % spin-label with extracted membrane lipids in (2:1 v/v) $CHCl_3/CH_3OH$ prior to removal of the organic solvent and dispersion of the lipid in buffer.

ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples were contained in 1-mm diameter sealed glass capillaries within standard 4-mm diameter quartz tubes containing light silicone oil for thermal stability. Spectral digitization and processing were performed by using a PDP 11/10 dedicated computer and a Digital Equipment Corp. LPS system with VT-11 display. Spectral subtraction was carried out by using interactive software written by Dr. W. Möller of the Max-Planck-Institut für biophysikalische Chemie. Further details of the ESR and spin-labeling techniques are given in Marsh (1982).

Results

The ESR spectra of the various C-14 nitroxide phospholipid labels in (Na^+,K^+)-ATPase membranes, at 4 °C, are given in Figure 1. Each of the spectra is composed of two components, corresponding to distinct lipid environments. One component corresponds to the fluid lipid environment found in aqueous dispersions of the extracted membrane lipids (spectra not shown). The second component (indicated by the arrows in Figure 1) has a larger splitting, is not seen in the spectra from the lipids alone, and represents a lipid environment with considerably more restricted mobility. This motionally restricted component is attributed to lipids interacting directly with the protein [c.f. Marsh & Watts (1982)]. From Figure 1, it is clear that the relative proportions of the fluid and motionally restricted lipid components vary considerably between the different spin-labels, indicating a marked degree of selectivity in the interaction with the (Na^+,K^+)-ATPase.

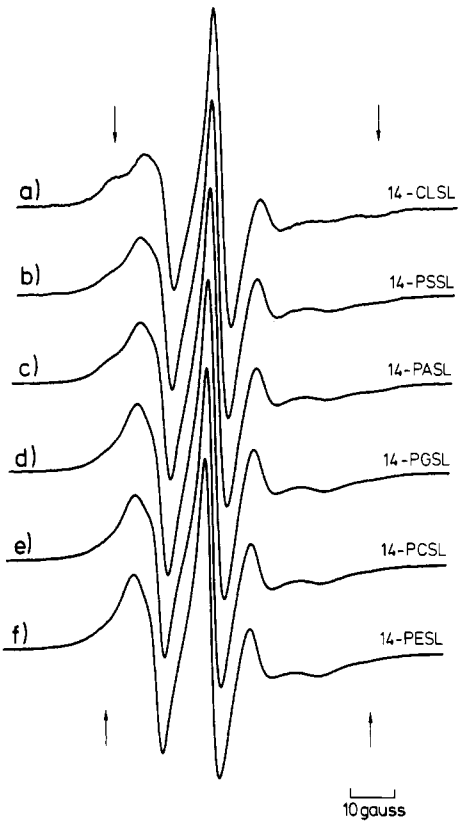

FIGURE 1: ESR spectra of the C-14 nitroxide spin-labels in (Na^+,K^+)-ATPase membranes from *Squalus acanthias* rectal gland, T = 4 °C: (a) diphosphatidylglycerol (cardiolipin) spin-label; (b) phosphatidylserine spin-label; (c) phosphatidic acid spin-label; (d) phosphatidylglycerol spin-label; (e) phosphatidylcholine spin-label; (f) phosphatidylethanolamine spin-label.

The ESR spectra of the various C-16 nitroxide labels in (Na^+,K^+)-ATPase membranes, at 0 °C, are given in Figure 2. Again, the spectrum of each label consists of a motionally restricted component, indicated by the arrows, in addition to the fluid lipid component. The spectra from aqueous dispersions of the extracted membrane lipids similarly consist solely of the fluid lipid component (spectra not shown). There also seems to be some selecitvity in the ratio of fluid to motionally restricted components, although it does not appear so marked as with C-14 labels.

The ESR spectra of the C-14 nitroxide stearic acid spin-label, 14-SASL, and the androstanol spin-label, ASL, in (Na^+,K^+)-ATPase membranes and in aqueous dispersions of the extracted membrane lipids are given in Figure 3. A motionally restricted component is clearly visible in the membrane spectra of the 14-SASL stearic acid label, which is not present in the spectrum of the membrane lipids alone. The membrane spectra of the androstanol label also contain a more motionally restricted component, in addition to the fluid component seen in the lipid spectra. The line shapes of ASL are rather different, since this label is optimally sensitive to long-axis rotation [cf. Marsh (1980, 1981)], but the more motionally restricted component is clearly seen in the expanded section of the high-field region of the membrane spectra.

The method of analyzing the two-component membrane spectra by digital subtraction is illustrated for the spectra of the C-14 nitroxide phosphatidylserine spin-label in Figure 4. A motionally restricted spin-label component (indicated by the arrows in Figure 4a) is clearly seen in the outer wings of the membrane spectrum and is not present in the spectrum from the lipids alone. Subtraction of the lipid spectrum from the membrane spectrum yields the spectrum of the motionally

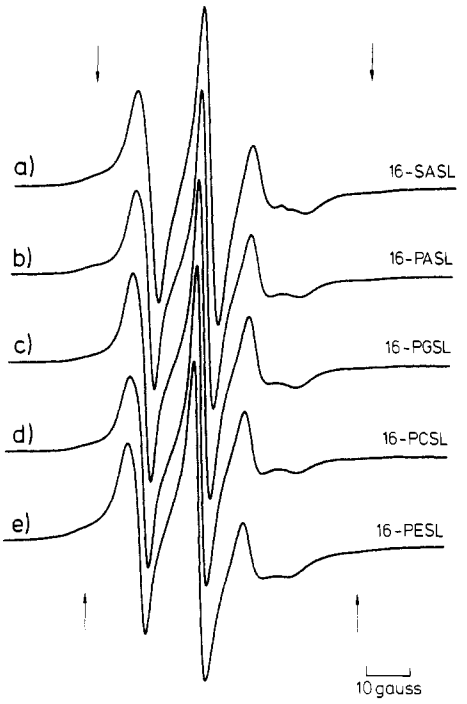

FIGURE 2: ESR spectra of the C-16 nitroxide spin-labels in (Na^+,K^+)-ATPase membranes from *Squalus acanthias* rectal gland, $T = 0$ °C: (a) stearic acid spin-label; (b) phosphatidic acid spin-label; (c) phosphatidylglycerol spin-label; (d) phosphatidylcholine spin-label; (e) phosphatidylethanolamine spin-label.

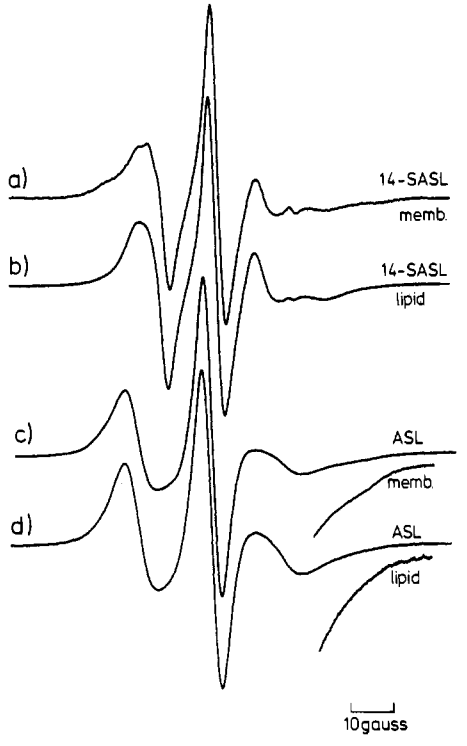

FIGURE 3: ESR spectra of the C-14 stearic acid spin-label 14-SASL and the androstanol spin-label ASL in (Na^+,K^+)-ATPase membranes and aqueous dispersions of extracted membrane lipids: (a) 14-SASL in membranes, $T = 4$ °C; (b) 14-SASL in lipids, $T = 4$ °C; (c) ASL in membranes, $T = 12$ °C; (d) ASL in lipids, $T = 12$ °C.

restricted component (Figure 4d), and double integration gives the fraction of the total intensity (37%) which is present in this component. The difference spectrum in Figure 4d is approaching the limit of motional sensitivity of conventional ESR [see, e.g., Marsh (1981, 1982)] and indicates a considerably reduced mobility relative to that of the fluid bilayer

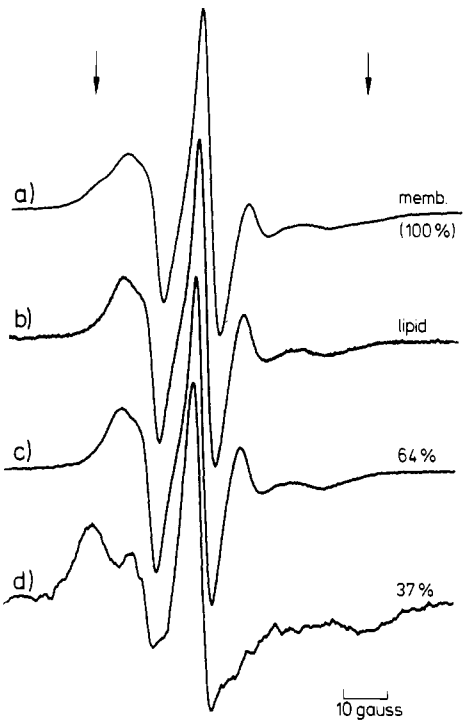

FIGURE 4: ESR difference spectroscopy of the C-14 phosphatidylserine spin-label 14-PSSL in (Na^+,K^+)-ATPase membranes, $T = 0$ °C: (a) membrane spectrum; (b) aqueous lipid dispersion spectrum; (c) fluid component difference spectrum obtained by subtracting a suitable immobilized spectrum (36% relative intensity) from the membrane spectrum; (d) immobilized component difference spectrum obtained by subtracting the lipid spectrum (63% of the double-integrated intensity) from the membrane spectrum.

Table I: Selectivity of C-14 Lipid Spin-Labels in (Na^+,K^+)-ATPase Membranes, $T = 0$ °C[a]

lipid	f	K_r^L/K_r^{PC}
CL*	0.52	3.8
PS*	0.33	1.7
PA*	0.30	1.5
PG*	0.21	0.9
SA*	0.33	1.7
PE*	0.21	0.9
PC*	0.22	1.0

[a] f = fraction of motionally restricted lipid; typical error in f is ±0.02. K_r = relative association constant. CL* is 14-CLSL, PS* is 14-PSSL, etc.

lipids. (It will be shown below that the line widths and line splittings of this difference spectrum may be used to give an estimate of the degree of motional restriction of the lipid chains by the protein.) Subtraction of the motionally restricted component from the membrane spectra, using a matching spectrum from dimyristoylphosphatidylcholine vesicles in the gel phase, yields the spectrum of the fluid lipid component (Figure 4c) which is very closely similar to that of the extracted lipids. Double integration yields a relative intensity of 64% for this fluid component, in agreement with the quantitation from the motionally restricted component.

High-quality difference spectra of the type indicated in Figure 4 are obtained at low temperatures, indicating that the membrane spectra can be accurately decomposed into two independent components. The fractions, f, of motionally restricted component obtained by the two complementary subtraction methods are given in Table I. These results indicate a preferential association with cardiolipin (CL), phosphatidic acid (PA), phosphatidylserine (PS), and stearic acid (SA) but no selectivity for phosphatidylcholine (PC), phosphatidyl-

Table II: Thermodynamic Parameters of Lipid Spin-Label Head-Group Selectivity in (Na^+,K^+)-ATPase Membranes, $T = 0$ °C

lipid	$\Delta G°_L - \Delta G°_{PC}$ (cal/mol)	$\Delta H°_L - \Delta H°_{PC}$ (cal/mol)	$\Delta S°_L - \Delta S°_{PC}$ (cal mol^{-1} K^{-1})
CL*[a]	−730	−240 ● 260	2.2 ± 1.0
PS*	−300	−820 ± 230	−1.5 ± 0.8
PA*	−230	−1510 ± 410[b]	−4.3 ± 1.5[b]
PG*	30	−2380 ± 480[b]	8.8 ± 1.8[b]
SA*	−300	−3360 ± 370[b]	−10.8 ± 1.4[b]
PE*	30		
PC*	0	0	0

[a] Lipids in this column are as defined in Table I. [b] Values are probably artifactually high; see text.

ethanolamine (PE), or phosphatidylglycerol (PG). Subtractions for the steroid label ASL yield a value of $f \sim 0.16$–0.21 at 16 °C, indicating little selectivity, or a slight negative selectivity, relative to phosphatidylcholine. Since the phospholipid composition of the (Na^+,K^+)-ATPase membranes consists mostly of the nonselective phosphatidylcholine and phosphatidylethanolamine (Esmann et al., 1980), the selectivity can be expressed in terms of the previously used equation for preferential occupancy of sites on the surface of the protein (Brotherus et al., 1981; Knowles et al., 1979):

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

where $n_f^*/n_b^* = (1-f)/f$ is the ratio of double-integrated intensity in the fluid and motionally restricted spin-label components, respectively, n_t is the total lipid/protein ratio in the membrane, N_1 is the total number of lipid sites directly interacting with the protein (first shell sites), and K_r is the association constant of the spin-label with these sites, relative to that of the background host lipid. If it is assumed that the total number of sites is the same for each lipid, then the selectivity between two lipids, A and B, can be expressed in terms of the ratio of the respective association constants, K_r^A/K_r^B (Knowles et al., 1981):

$$\frac{(n_f^*/n_b^*)^A}{(n_f^*/n_b^*)^B} = \frac{K_r^B}{K_r^A} = \frac{K^B}{K^A} \quad (2)$$

These values are given relative to the phosphatidylcholine spin-label 14-PCSL in Table I. The effective free energies of association of the two lipids with the first shell sites on the protein are then given by

$$\Delta G°_A - \Delta G°_B = -RT \ln (K^A/K^B) \quad (3)$$

The effective free energies of association relative to the 14-PCSL label are given in Table II.

The spectral line shapes of the various lipid spin-labels in the (Na^+,K^+)-ATPase membranes change considerably with temperature as indicated in Figure 5. The largest changes occur in the fluid component, which gets considerably sharper as the temperature increases. For this reason, the fluid component begins to dominate over the motionally restricted component at the higher temperatures. Under these conditions, the spectral subtractions to obtain the relative proportions of the two components become considerably more difficult. The protocol adopted in performing the subtractions was the following: (1) The extracted lipid spectrum which corresponded most closely to the fluid component in the membrane spectrum was taken; usually this was the spectrum from lipids at the same temperature as the membranes, but occasionally it was ~2 °C different. (2) The lipid spectrum was subtracted from the membrane spectrum to give a motionally restricted difference spectrum which most closely corresponded to one of a series of trial spectra from the 14-PCSL label in sonicated

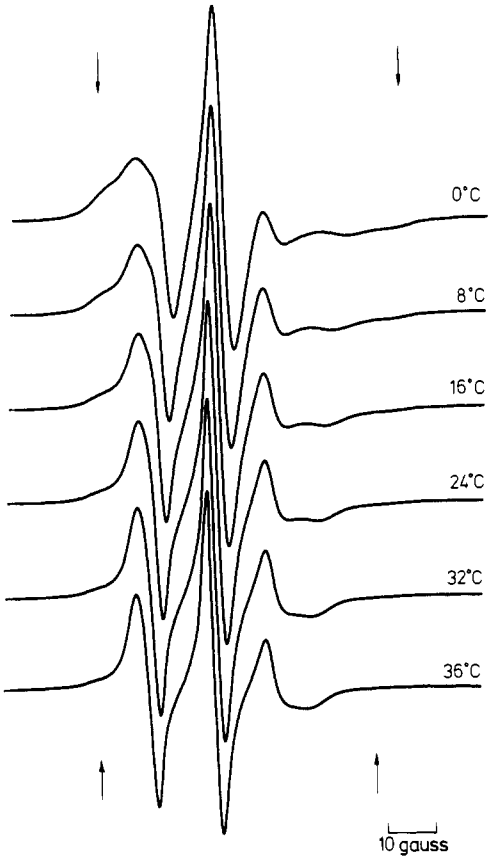

FIGURE 5: Temperature dependence of the ESR spectra of the C-14 phosphatidic acid spin-label 14-PASL in (Na^+,K^+)-ATPase membranes.

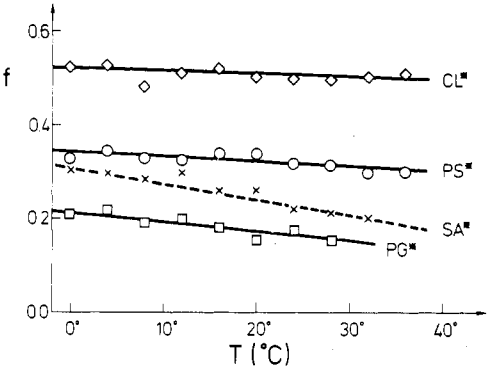

FIGURE 6: Temperature dependence of the fraction of motionally restricted lipid deduced from the ESR spectra of C-14 lipid spin-labels in (Na^+,K^+)-ATPase membranes. CL*, 14-CLSL; PS*, 14-PSSL; SA*, 14-SASL; PG*, 14-PGSL.

dimyristoylphosphatidylcholine (DMPC) vesicles at various temperatures in the gel phase. This spectrum was used in assessing the end point. (3) The selected DMPC spectrum was then used to subtract from the membrane spectrum to give a fluid difference spectrum whose end point was determined by comparison with the lipid spectrum selected in (1). In general, the two complementary methods of subtraction gave comparable values for f, although the subtraction in (3) was the more reliable. The one exception was with the 14-CLSL label which gave values consistently 10% higher for the subtraction method described in (2) because of a second, minor fluid component which was not allowed for by this method.

The temperature dependence of the fraction of motionally restricted lipid, f, is given for the various labels in Figure 6. These values are the means of the two subtraction methods, except for cardiolipin where the methods differ significantly

Table III: Effective Rotational Correlation Times (τ_R) for the Motionally Restricted Spin-Labeled Lipid Component in (Na^+,K^+)-ATPase Membranes, T = 0 °C[a]

spin-label	$\tau_R(A_{max})$ (ns)	$\tau_R(\Delta H_l)$ (ns)	$\tau_R(\Delta H_h)$ (ns)
14-CLSL	28	30	33
14-PSSL	42	33	54
14-PASL	49	28	47
14-SASL	38	28	40

[a] $\tau_R(A_{max})$, $\tau_R(\Delta H_l)$, and $\tau_R(\Delta H_h)$ are deduced from the outer hyperfine splitting, the extreme low-field line width, and the extreme high-field line width, respectively.

and the value obtained from method 3 above is given. It is seen from Figure 6 that with the exception of the stearic acid label, 14-SASL, which is anomalous, there is only a very small decrease in f with increasing temperature. For the phosphatidylcholine label 14-PCSL, there is no significant temperature dependence at all. Although there is considerable scatter on the values of f, the temperature dependence can be used to obtain further thermodynamic data relating to the lipid specificity. If $\Delta H°$ is the excess enthalpy associated with the differential lipid–protein interaction, then

$$\Delta H°_A - \Delta H°_B = -R \frac{\partial \ln (K_r^A/K_r^B)}{\partial(1/T)} \quad (4)$$

or if 14-PCSL, which has very little temperature dependence, is chosen as the comparison lipid, this can be reduced to $\Delta H°_L - \Delta H°_{PC} = -R[\partial (\ln K_r^L)/\partial(1/T)]$. Correspondingly, the values for the excess entropy associated with the lipid selectivity are obtained from $\Delta G°_A - \Delta G°_B = \Delta H°_A - \Delta H°_B - T(\Delta S°_A - \Delta S°_B)$. Values for all three thermodynamic parameters obtained from the temperature dependences and the selectivities at T = 0 °C are given for the various labels in Table II. The enthalpies for the cardiolipin and phosphatidylserine labels are rather small, comparable to the values of the free energy of association. The apparent enthalpies for phosphatidic acid and especially stearic acid are considerably higher, but it is likely that this may be artifactual since it has subsequently been found (Esmann & Marsh, 1985) that both lipids lie within their titration range for the protein–lipid interaction at pH 7.4. The phosphatidylglycerol label also has a steeper temperature dependence and a correspondingly larger effective enthalpy, but for this label, the values of f are rather small and thus are more difficult to measure accurately.

Spectral subtractions as shown in Figure 4 also allow analysis of the dynamic properties of the two lipid components. For the motionally restricted component (cf. Figure 4d), the spectra may be analyzed in terms of the splittings A_{zz}', and line widths, $\Delta H_{l,h}$, of the outer hyperfine lines according to the slow-motion simulations of Freed (1976). In the slow-motional regime ($\tau_R \sim$ 10–100 ns), increasing rates of rotational motion are accompanied by a decrease in splitting and an increase in line widths of the outer hyperfine lines. Empirical correlation time calibrations based on isotropic motion can be used: $\tau_R = a(1 - A_{zz}'/A_{zz}^R)^b$ and $\tau_R = a_m'(\Delta H_m/\Delta H_m^R - 1)^{b_m'}$, where A_{zz}^R and ΔH_m^R are the corresponding rigid limit values of the outer hyperfine splitting and line widths, respectively. In the case of anisotropic motion, these calibrations will approximate the correlation time for rotation of the nitroxide z axis (Freed, 1976). Effective correlation times at 0 °C are given in Table III for those spin-labels which yield high-quality difference spectra. For spin-labels with no specificity, the motionally restricted components are small, and an insufficient signal to noise ratio is obtained in the difference spectra. The values of the effective correlation time lie within

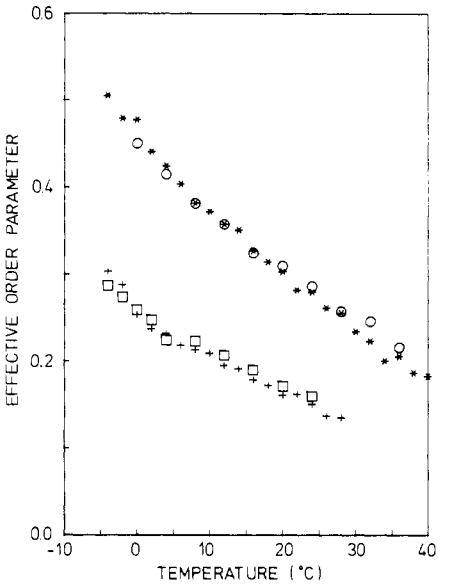

FIGURE 7: Effective order parameter of the fluid lipid component for the phosphatidylserine spin-label 14-PSSL in (Na^+,K^+)-ATPase membranes (○) and extracted lipid dispersions (*) and for the phosphatidic acid spin-label 16-PASL in membranes (□) and extracted lipid dispersions (+).

the range $\tau_R \sim$ 30–50 ns for all labels. This indicates a very significant slowing down of the motion relative to that of the fluid lipid component, which has correlation times approximately in the range $\tau_R \sim$ 1–5 ns. With increasing temperature, as the fluid component begins to dominate the spectrum, the scatter on the data from the motionally restricted component is too great to determine a reliable temperature dependence.

The fluid component difference spectra (cf. Figure 4c) have been analyzed in terms of the conventional order parameter formalism and compared with results from the extracted membrane lipids in Figure 7. Order parameters were calculated from

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \frac{a_0'}{a_0} \quad (5)$$

where $2A_{\parallel}$ is equal to the outer, maximum hyperfine splitting ($2A_{max}$) and $A_{\perp}$ is obtained from the inner, minimum hyperfine splitting ($2A_{min}$) according to (Gaffney, 1976): $A_{\perp}$ (G) = A_{min} (G) + 1.4{1 − ($A_{\parallel} - A_{min}$)/[$A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})$]}. The isotropic hyperfine splitting constant is given by

$$a_0 = \frac{1}{3}(A_{\parallel} + 2A_{\perp}) \quad (6)$$

and that corresponding to the single crystal environment in which the principal values of the hyperfine tensor A_{xx}, A_{yy}, and A_{zz} were measured (Jost et al., 1971) is given by $a_0' = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$. However, because the spectra of the fluid lipid component are approaching the slow-motion regime, for which the spectral splittings depend on the rate of motion as well as its amplitude, the values calculated according to eq 5 must be considered as effective values. It is seen from Figure 7 that the effective order parameters of the fluid lipid component in the membrane are closely similar to those of dispersions of the extracted membrane lipids for both lipids labeled at the C-14 atom position and lipids labeled at the C-16 atom position throughout the whole temperature range studied. Similar results are obtained for the other labels not shown in Figure 7. Values of the isotropic hyperfine splitting factors in the membranes are compared with those from the extracted lipids for a variety of labels in Table IV. The measured values

Table IV: Mean Values of the Isotropic Hyperfine Splitting Constant, a_0 (G), for Lipid Spin-Labels in (Na^+,K^+)-ATPase Membranes and Dispersions of the Extracted Membrane Lipids

label	a_0 (G) membrane	a_0 (G) lipid
14-SASL	14.06 ± 0.06	14.06 ± 0.06
14-PASL	14.13 ± 0.07	14.12 ± 0.03
14-PSSL	14.20 ± 0.05	14.08 ± 0.02
14-PCSL	14.18 ± 0.06	14.10 ± 0.04
14-PGSL	14.09 ± 0.04	14.11 ± 0.06
14-PESL	14.10 ± 0.08	14.16 ± 0.09
14-CLSL	14.12 ± 0.04	14.03 ± 0.09
16-SASL	14.06 ± 0.16	14.08 ± 0.07
16-PASL	14.09 ± 0.08	14.07 ± 0.04
16-PCSL	14.09 ± 0.08	14.07 ± 0.07
16-PGSL	14.09 ± 0.05	14.07 ± 0.08
16-PESL	14.00 ± 0.05	14.02 ± 0.01

remained constant with changing temperature throughout the range in Figure 7 as required, since a_0 should be sensitive to environmental polarity, but not to motion. This gives some confidence in the values derived for the order parameters, but because of slow motional effects they must nonetheless be considered only as effective values. Within experimental accuracy, the a_0 values in Table IV are identical in membranes and extracted lipids and are similar for all labels studied.

Discussion

A motionally restricted spin-labeled lipid component is observed in (Na^+,K^+)-ATPase membranes in addition to the normal fluid lipid component seen in dispersions of the extracted membrane lipids. This second component is seen with all labeled lipids studied and is attributed to the lipids interacting directly with the integral membrane proteins, in this case predominantly the (Na^+,K^+)-ATPase. The fraction of motionally restricted lipid is least for the phosphatidylcholine spin-label among others, and phosphatidylcholine is also the major lipid in the membrane (Esmann et al., 1980). Therefore, it seems reasonable to assume that the phosphatidylcholine label displays no preferential selectivity for the protein, i.e., $K_r(PC^*/PC) = 1$ in eq 1, and this label can be used to estimate the number of lipid sites, N_l, associated with the protein. The total phospholipid/protein ratio in the membrane is 300/1 mol/mol, assuming an average phospholipid molecular weight of 760 and taking the molecular weight of the $\alpha_2\beta_2$ (Na^+,K^+)-ATPase dimer to be 265 000 (Esmann et al., 1980). With a fraction of motionally restricted lipids $f = 0.22 \pm 0.02$, this gives a value of $N_l = 66 \pm 6$ lipid sites per 265 000-dalton (Na^+,K^+)-ATPase. This estimate ignores the cholesterol content of the membrane. The rationale for doing this is the well-known condensing effect of cholesterol (de Bernard, 1958; Marsh & Smith, 1973) which causes the cholesterol molecule to occupy interstitial rather than substitutional sites in the fluid lipid bilayer. Clearly this assumption introduces some uncertainty into the measured stoichiometry, but the results of lipid–protein titrations of reconstituted (Na^+,K^+)-ATPase have yielded a value of $N_l = 63 \pm 3$ lipid sites per 314 000-dalton protein (Brotherus et al., 1981), in substantial agreement with the present value.

To obtain some structural interpretation of this effective lipid/protein stoichiometry, it is of interest to compare the stoichiometry with the known dimensional data on the protein. Negative-stain electron microscopy and freeze–fracturing electron microscopy have suggested that the (Na^+,K^+)-ATPase is present in the membrane as the $\alpha_2\beta_2$ dimer, with a maximum intramembranous diameter for the $\alpha\beta$ monomer of 50 Å (Deguchi et al., 1977). A projection map of two-dimensional negatively stained protein crystals of the (Na^+,K^+)-ATPase has been obtained to a resolution of 25 Å by electron microscopy (Hebert et al., 1982). These latter studies also confirm that the cross-sectional diameter of the $\alpha\beta$ monomer is ~40–60 Å. Assuming a lipid chain diameter of 4.8 Å, it can be calculated that approximately 36 lipid molecules can be situated around a perimeter 2.4 Å out from the surface of a protein monomer cylinder of 50-Å diameter (allowing for two chains per phospholipid and both halves of the bilayer). Thus, a maximum of 72 lipids can be accommodated around the dimer if there is lipid in the intervening region between the two monomers. This value decreases to approximately 57 lipids per dimer if the region between the two monomers is inaccessible to lipid, and this interface is approximated by two planar surfaces tangential to the two protein cylinders. Clearly, the values for the numbers of motionally restricted lipids observed by spin-label spectroscopy lie within this range, suggesting that the motionally restricted lipids may form a continuous boundary shell around the dimeric protein complex. However, it should be emphasized that the exact surface contour of the protein is not yet known. Thus, it cannot be excluded that the motionally restricted lipids are accommodated within surface invaginations rather than covering the entire lipid–protein interface.

A functional significance for this stoichiometry of the motionally restricted lipid component is also suggested from experiments on delipidation of the enzyme in the poly(oxyethylene) ether detergent $C_{12}E_8$. Optimal activity is maintained down to phospholipid contents of 0.19 mg/mg of protein, and below this level, activity is progressively lost even in the presence of excess detergent (Esmann, 1984). Thus, approximately 66 phospholipid molecules per 265 000-dalton protein are required for fully activity, which correlates rather well with the stoichiometry of the motionally restricted lipid associated with the protein.

The experiments with the different spin-labeled phospholipids demonstrate a clear head-group specificity in the association of the motionally restricted lipids with the protein. The pattern of selectivity differs from that observed previously with other proteins [see, e.g., Marsh (1983, 1985)] in that cardiolipin has the highest specificity of the lipids tested, rather than stearic acid as is found for the myelin proteolipid protein (Brophy et al., 1984), and phosphatidic acid does not have a higher specificity than phosphatidylserine as is found for cytochrome oxidase (Knowles et al., 1981) or the acetylcholine receptor (Ellena et al., 1983). More strikingly, essentially no specificity is observed in the association of the same spin-labeled lipids with rhodopsin (Watts et al., 1979; Marsh et al., 1982). This comparison demonstrates that the selectivity is not solely a property of the lipids but depends, as one might expect, on the detailed amino acid configuration of the protein. Although the specificity is greatest for the negatively charged lipids, it does not bear a direct relation to the net charge on the lipid headgroup. In particular, no preferential association is observed for phosphatidylglycerol which has the same net negative charge as phosphatidylserine or stearic acid. Thus, the pattern of lipid specificity is not solely electrostatic in origin but presumably depends also on head-group hydration, hydrogen bonding, and other chemical effects. More recent experiments on the pH and salt dependence of (Na^+,K^+)-ATPase specificity have shown that electrostatic effects can account for most of the specificity observed with stearic acid but only a small part of that found with phosphatidic acid and phosphatidylserine (Esmann & Marsh, 1985). These results are in accord with the previous finding that the specificity of

other, both positively and negatively charged, *single tail* lipids is of electrostatic origin (Brotherus et al., 1980). Clearly, the interactions with glycerophospholipids are more complex.

A selectivity for spin-labeled phosphatidic acid and phosphatidylserine has also been demonstrated by the reduced spin–spin interaction in (Na^+,K^+)-ATPase membranes from *Torpedo marmorata* (Zachowski & Devaux, 1983). These results are interesting since they also suggest a reduced rate of lipid exchange at the protein interface (cf. below). The selectivity for certain negatively charged phospholipids may also have a functional significance, since several studies have found a more efficient reconstitution of activity in the presence of acidic lipids (Kimelberg & Papahadjopoulos, 1974; Wheeler et al., 1974; Palatini et al., 1977; Mandersloot et al., 1978; Cornelius & Skou, 1984). However, in some of these cases phosphatidylglycerol, for which there is no specificity, was found to be effective and in other studies a nonspecific potentiation by negative charges was suggested (de Pont et al., 1978) and in yet a further study an active preparation was obtained with phosphatidylcholine alone (Hilden & Hokin, 1976).

The free energies associated with the lipid specificities given in Table II are relatively modest, of the same order as thermal energies. This could favor fine control of enzyme activity via site occupancy [see Sandermann & Gottwald (1983)] if a particular lipid had an activating or inhibitory role. The experimental uncertainty in the temperature dependence of the fraction of motionally restricted lipid, f, severely limits the accuracy of the determination of the enthalpic contributions to the free energies. This is particularly the case for lipids with low values of f such as phosphatidylglycerol. Unfortunately, it has also been found subsequently that at pH 7.4, corresponding to maximum activity of the enzyme, both phosphatidic and stearic acids are within their acid–base titration regions for the protein–lipid interaction (Esmann & Marsh, 1985). Thus, the temperature dependence of f for these two lipids is artifactually high due to the temperature dependence of the buffer. Therefore, of the results in Table II, only those for cardiolipin and phosphatidylserine can be expected to be reasonably reliable. The enthalpic contributions for these two lipids are of the same order of magnitude as the total free energy and, within the error limits, are of sufficient size to account for nearly all of the selectivity in the free energy of association. Certainly there is no extensive entropy–enthalpy compensation involved in the specificity of interaction.

Essentially no specificity is observed for the spin-labeled steroid molecule androstanol (ASL) relative to phosphatidylcholine. This may well be relevant to the behavior of cholesterol in the membrane. A similar finding has also been made for cytochrome oxidase (Jost et al., 1973) and for rhodopsin (Watts et al., 1979). However, by contrast, there is a positive selectivity in the interaction of androstanol with the acetylcholine receptor (Ellena et al., 1983) and a negative one for its interaction with the myelin proteolipid protein (Brophy et al., 1984). There is also a negative selectivity in the interaction of a spin-labeled cholesterol analogue with the Ca^{2+}-ATPase from sarcoplasmic reticulum (Silvius et al., 1984). It should also be pointed out that, in spite of certain mechanistic and structural similarities between the two ATPases, there is very little similarity in the patterns of lipid specificity, since the Ca^{2+}-ATPase shows relatively little selectivity between the various phospholipids (C. Hidalgo and D. Marsh, unpublished results).

The line shapes in the ESR difference spectra give information on the dynamics of the lipid–protein interactions. Unfortunately, it was not possible to obtain reliable data on the temperature dependence of the difference spectra of the motionally restricted component, which would have been diagnostic of motion within the slow-motion regime. However, analysis of the line shapes at 0 °C strongly suggests that the motionally restricted spin-labeled lipids have effective rotational correlation times in the range 30–50 ns. By comparison, the fluid lipid component has rotational correlation times in the range 1–5 ns (Schindler & Seelig, 1973; Lange et al., 1985). Thus, the motion of the lipid chains is significantly slowed down by interaction with the hydrophobic surface of the protein. There are two motions which can contribute to the effective correlation time of these lipids interacting directly with the protein. These are motions of the lipid chains in contact with the protein and the exchange of the lipids on and off the protein surface. Thus, a *lower* limit for the lifetime of the lipids on the surface of the protein is given by the effective rotational correlation time for the motionally restricted lipids quoted above, i.e., $\tau_{off} \gtrsim \tau_R \sim 30$–$50$ ns. This is somewhat shorter than the average lipid–lipid exchange lifetime in fluid lipid bilayers, resulting from lateral diffusion, which is given in terms of the lateral diffusion coefficient, D_{lat}, and the mean square distance, $\langle x^2 \rangle$, between diffusive jumps by $\tau_{diff} \sim \langle x^2 \rangle / 4D_{lat} \sim 100$–$200$ ns (Träuble & Sackmann, 1972; Devaux et al., 1973). Thus, it seems likely that there is some independent lipid motion on the surface of the protein, in addition to exchange. A *lower* limit for the exchange lifetime is given by the difference in spectral splittings ($H_f - H_b$) of the fluid and motionally restricted lipid populations, since this must be greater than the exchange frequency for independent spectral components to be observed. From the smallest splittings (between the low-field peaks), it can be estimated that $\tau_{on,off} \gtrsim \hbar(H_f - H_b)^{-1} \sim 50$ ns. A direct estimate of the exchange lifetime of the motionally restricted lipids can be made from the increase in the first-derivative line width, δH_{pp}, of the fluid lipid component in membranes relative to that in the extracted lipids, since the former is subject to lifetime broadening resulting from exchange (Marsh et al., 1982). The off rate constant is then given by $\tau_{off}^{-1} = (\sqrt{3}/2)(g\beta/\hbar)\delta H_{pp}(1-f)/f$ [see, e.g., Marsh (1985)]. From Figure 7, it is seen that the spectral characteristics of the fluid membrane lipids are very similar to those of the extracted lipids. The line widths are also practically identical within experimental accuracy. Thus, the exchange of lipids off the protein is likely to be slow. However, a typical experimental uncertainty of 0.3 G in δH_{pp} would give rise to a lower limit for the exchange lifetime of $\tau_{off} \gtrsim 20$ ns for phosphatidylcholine and 70 ns for cardiolipin, which is within the range for free lateral diffusion of lipids in fluid bilayers. In addition to indicating little coupling by exchange, the results of Figure 7 also indicate that the perturbation of the lipids by the protein is short range, since very little difference is seen in the fluid lipid component in membranes and extracted lipids. The isotropic hyperfine splitting constants in Table IV, besides indicating the consistency of the analysis, demonstrate that the spin-labels are incorporated into the membranes in the same way as into lipid bilayers. Both the 14- and 16-position labels record a low polarity, characteristic of the center region of cholesterol-containing bilayers (Marsh & Watts, 1981; R. D. Pates et al., unpublished results).

In summary, the spin-label studies on (Na^+,K^+)-ATPase membranes have revealed that, in common with several other systems, the lipid–protein interactions are of short range, approximating one shell around the protein. The stoichiometry of the motionally restricted lipids interacting directly with the

protein correlates rather well with the number of phospholipids required for activity. The protein displays an interesting pattern of lipid specificity which correlates to some extent with the efficiency of negatively charged lipids in supporting activity.

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Registry No. ATPase, 9000-83-3; ASL, 25521-33-9; stearic acid, 57-11-4; androstanol, 58855-92-8.

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