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Charge selectivity at the lipid–protein interface of membranous Na,K-ATPase

(boundary layer/membrane enzyme/spin labeling/electron spin resonance)

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ABSTRACT **Lipid interactions with the integral membrane protein Na,K-ATPase (ATP phosphohydrolase, EC 3.6.1.3) purified from the electric organ of *Electrophorus electricus* were studied by spin labeling. A protein-associated component (boundary layer) in equilibrium with the fluid bilayer is clearly evident in the electron spin resonance spectra. The influence of charge on this equilibrium was determined by varying the head group of the lipid while maintaining the chain length and the position of the label constant. The lipid spin labels were 14-proxylstearylmethyl phosphate and the corresponding dimethylphosphate, alcohol, and quaternary amine. By using a pairwise spectral analysis, as well as a conventional spectral analysis, the binding affinity was found to decrease in the order of negative > neutral > positive charges. The fraction bound decreased from about 0.57 for the negatively charged phosphate to 0.25 for the positively charged quaternary amine. The amount of each bound lipid was nearly constant over the temperature range investigated (5–35 °C). High salt concentrations reversibly abolished the selectivity between the labels, confirming the role of charge in the binding equilibria.**

Na,K-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is a transmembranous protein involved in the active transport of Na^+ and K^+ in animal cells (1). All active Na,K-ATPase preparations contain phospholipids, and delipidation by detergents or phospholipase digestion inactivates the enzyme. The activity often can be restored partially by adding lipids (for review, see ref. 2). Anionic lipids, including several detergents (3–8), reactivate lipid-depleted Na,K-ATPase the most efficiently. Cationic lipids have not been observed to reactivate the enzyme (6–8); conflicting results have been obtained with neutral phospholipids (2).

These enzyme activity measurements provide important information on the effects of lipids at the functional level. However, there is little information available on the corresponding structure and organization of protein and lipids at the molecular level. The activation by negatively charged lipids could be caused indirectly by altering the properties of the bilayer or directly by selective interactions with the protein. In order to test for direct interactions, we have examined membranous preparations of purified Na,K-ATPase from the electric eel by using lipid spin labels with polar head groups of different charges. Electron spin resonance (ESR) provides a means of distinguishing between protein-associated lipid label and the fluid bilayer component (9). Our results show that there is a definite preference for the negatively charged lipid at the lipid–protein interface. The temperature dependence and the origin of this charge preference are examined.

MATERIALS AND METHODS

Sample Preparation. Na,K-ATPase was purified from the electric organ of the electric eel *Electrophorus electricus* (10) and had a specific activity of 25 μmol of P_i/min per mg of protein. Of the protein staining with Coomassie blue on sodium dodecyl sulfate/polyacrylamide gels, 90–95% was accounted for by the catalytic and the glycoprotein polypeptides of the Na,K-ATPase. The remaining 5–10% of the protein was accounted for by a protein band, near the tracking dye, with a molecular weight of 10,000. The residual content of Lubrol WX was decreased by dialysis at 5°C against Amberlite XAD-2 resin in 0.25 M sucrose/1 mM Tris/1 mM EDTA/0.5 mM dithiothreitol, pH 7.3, and then against the same buffer without dithiothreitol. The phospholipid (11) to protein (12) weight ratio of the preparation was 0.32, assuming an average phospholipid molecular weight of 775 and using bovine serum albumin as the protein standard. Partially lipid-depleted Na,K-ATPase was prepared by treating the purified enzyme (4.6 mg) with Lubrol WX (50 mg) for 60 min at 0°C and sedimenting the sample (0.6 ml) into a 5–20% linear sucrose gradient. The fractions containing the protein peak were pooled and dialyzed to decrease the residual detergent content; the final phospholipid to protein ratio (wt/wt) was 0.13.

Spin-Labeling Techniques. The spin labels used were all derivatives of 14-proxylstearic acid in which the proxyl moiety (2,2-disubstituted 5,5-dimethylpyrrolidine-*N*-oxyl) (13) was at the C-14 position on the C_{18} chain, analogous to the well-known doxyl fatty acids (13). The lipid spin labels were sodium 14-proxylstearylmethyl phosphate (1) and the homologous dimethyl phosphate (2), 14-proxylstearyl alcohol (3), and 14-proxylstearyltrimethyl ammonium methanesulfonate quaternary amine (4). The details of the synthesis, purification, and characterization of these labels will be presented elsewhere. The spin labels were introduced into 1.4-mg samples of Na,K-ATPase by diffusion from a dry film at 0°C at a labeling level of less than 1 mol per 150,000 daltons of Na,K-ATPase, and the samples were then concentrated by centrifugation and sealed in capillaries. ESR spectra were recorded on a Varian E-line 9.5-GHz spectrometer at a microwave power of 5 mW with temperature maintained within 0.2°C. Spectra were digitized during collection and were treated as described (9) and also by an additional new method of data analysis described in the text.

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Abbreviations: ESR, electron spin resonance; proxyl, 2,2-disubstituted 5,5-dimethylpyrrolidine-*N*-oxyl.

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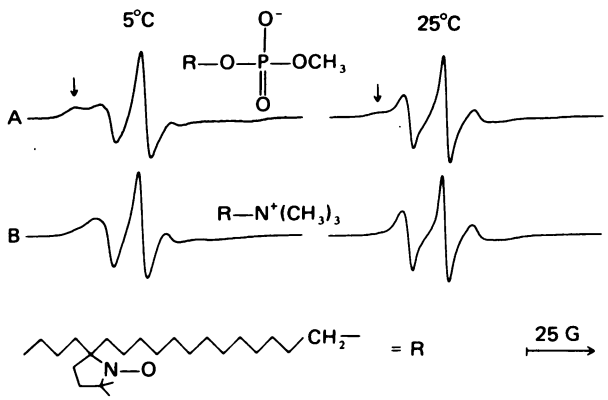

FIG. 1. Representative experimental ESR spectra obtained from identical aliquots of membranous Na,K-ATPase with (*A*) the negatively charged methyl phosphate lipid spin label (**1**) and (*B*) the positively charged quaternary amine spin label (**4**) at 5 and 25 °C. The arrows mark the position of the bound components clearly visible in the top spectra. *A* and *B* are the designations for experimental spectra used in the pairwise quantitative analysis (see text).

RESULTS

Identification of the Spectral Components. Representative ESR spectra of the negatively charged methyl phosphate (**1**) and positively charged quaternary amine (**4**) spin labels diffused into membranous Na,K-ATPase are shown in Fig. 1. These two labels gave rise to the largest spectral differences; the spectra of dimethyl phosphate (**2**) and the alcohol (**3**) were intermediate between these two extremes. We analyzed the spectra in two ways.

The first method (pairwise subtraction) does not involve the separate reference samples normally used in such analyses (9). It is made possible by using two spin labels that differ in their distributions between two presumptive environments of the sample. This method makes *no* assumptions about the line shapes in the two environments (except that they are clearly distinguishable), but it does assume that line shapes of the different labels in the same environments are the same, so that the experimental spectra are linear combinations of the two basic components. The unknown line shapes of the individual components are arrived at by pairwise subtraction of increasing increments of the digitized spectra of two different spin labels. Depending on the direction of this subtraction, the line shape of each component can be obtained directly. The judgment of the end point of the subtraction is based both on the emergence of a characteristic single-component line shape and on the maintenance of a zero baseline in the absorption spectrum (i.e., the first integral contains no significant negative values). Subtraction beyond these end points leads to easily recognized distortions in the spectra.

When this method was applied to the experimental spectra of Fig. 1, the result was the individual component line shapes a and b shown in Fig. 2. The broad line shapes (spectrum a) are essentially identical to the reference spectrum (c) obtained from a partially lipid-depleted sample and are characteristic of restricted motion of the label on the ESR time scale (9). This component was not observed in pure lipid bilayers but it was prominent in our Na,K-ATPase samples and has previously been observed in membranes containing other integral proteins (e.g., refs. 14–19). This component represents label that is in contact with the protein—i.e., in the boundary lipid layer. In principle, because of the method of introducing the lipid spin labels, it is possible that some fraction of the protein-associated lipid component is in a hydrophobic site separate from the lipid–protein interface. However, there is no evidence for such

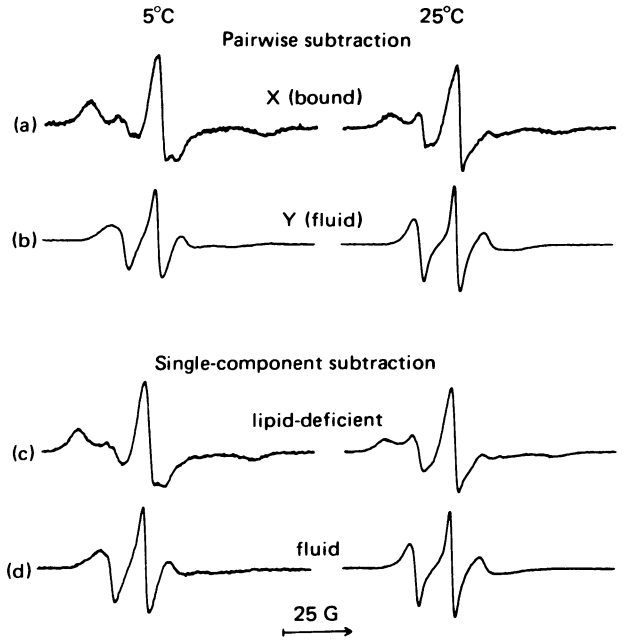

FIG. 2. Spectral components obtained from the experimental spectra in Fig. 1, resolved by two different methods. The top set (spectra a and b) were obtained by the pairwise subtraction method discussed in the text. X and Y are the designations used for these components in the quantitative analysis. In the single-component method, the spectrum of the partially lipid-depleted sample (c) is used as a bound component to obtain the difference spectrum (d) from the experimental spectra (Fig. 1, spectrum a).

a separate hydrophobic site in the Na,K-ATPase. The other spectral component (Fig. 2, spectrum b) is characteristic of a spin label in a more mobile environment (the lipid bilayer) (20, 21). Although it resembles the labels in vesicles of the electric organ lipids at the same temperature, the lines are broader, indicating somewhat slower motion in the bilayer environment of the Na,K-ATPase preparation, evidently due to the influence of the protein on the motion in the bilayer.

The second method used to analyze these data is the conventional method of single-component subtraction, requiring an additional reference sample of one of the spectral components. This may be either the bound component (14–17) or the mobile component (17). The bound line shape can be approximated by samples of low lipid content in which the motion of the label is greatly restricted (Fig. 2, spectrum c). Subtraction of increasing increments of the low-lipid reference spectrum until the end point is reached (9) gives the fraction of labels in each component directly from the integrated intensities of the spectra.

Quantitative Treatment of the Pairwise Difference Spectra. The method of pairwise subtraction can also be used to determine the relative concentrations of each component. Two experimental spectra, A and B, have integrated intensities a and b. Increasing amounts of spectrum B are subtracted from spectrum A to yield one spectral component, X, and the amount subtracted, u. Then A is subtracted from B until the other end point is reached, yielding the spectral component Y and the amount v. The integrated intensities x and y of the two endpoint spectra X and Y are given by $x = a - ub$ and $y = b - va$. Solving for a and b yields $a = (1 - uv)^{-1}x + u(1 - uv)^{-1}y$ and $b = v(1 - uv)^{-1}x + (1 - uv)^{-1}y$, in which the first term of each expression gives the contribution of X (e.g., the bound line shape) and the second term gives that of Y (e.g., the bilayer). The fractions of the bound spectrum F_A^x and F_B^x in the two composite spectra A and B are given by $F_A^x = (a - ub)/[a(1 -$

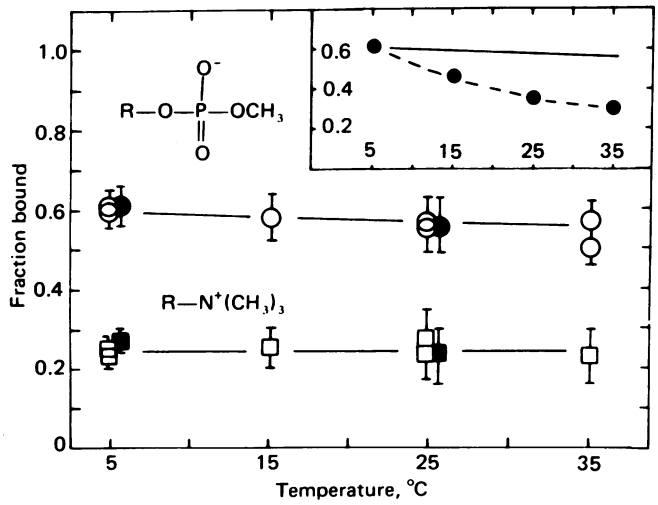

FIG. 3. Fraction of the negatively and positively charged lipid spin labels bound as a function of temperature. By the single-component subtraction method, the alcohol (2) and dimethyl phosphate (3) values were 0.34 and 0.41 at 5°C and 0.31 and 0.34 at 25°C. All single-component subtractions were corrected to compensate for the presence of 10% bilayer component in the spectra from the partially lipid-depleted sample. Vertical lines, range of systematic error introduced by calculations in both methods when values of end points are used that reflect deliberate over- and undersubtractions; solid symbols, single-component method; open symbols, pairwise subtraction method. ○, ●, Methyl phosphate (1); □, ■, quaternary amine (4). (*Inset*) Solid circles and dashed line show the artifactual decrease in the bound fraction of the methyl phosphate label (1) with increasing temperature, caused by using the 5°C bound reference sample for all single-component subtractions. The effect of neglecting the small changes in the bound spectrum as a function of temperature is clearly evident when compared to the appropriate spectral subtractions indicated by the solid line.

$uv)]$ and $F_B^x = [v(a - ub)]/[b(1 - uv)]$. The two remaining fractional values are given by $F_A^y = 1 - F_A^x$ and $F_B^y = 1 - F_B^x$.

Charge and Temperature Dependence of the Boundary–Bilayer Equilibrium. The fraction of the bound component obtained by the two methods described above is plotted in Fig. 3 as a function of temperature. The two methods of spectral analysis show good agreement, strengthening the basis of the two-environment model. It is interesting to note that the analysis shows that the spectra of the quaternary amine spin label (Fig. 1, spectrum b) contain about 25% of the bound component. This is not easily detected visually, even at 5°C. In fact, the spectra of the quaternary amine label generally look much like those of the bilayer component (Fig. 2, spectra b and d), although the lines are somewhat broader in the corresponding composite spectra of Fig. 1, spectrum b.

The spectral line shapes change as a function of temperature. This is principally due to the increased motion in the bilayer. There is also a small increase in the motion of the bound lipid. The distance between the high- and low-field extrema ($2A_{max}$) of the bound component measured from the bound *difference* spectra (pairwise subtraction method) were 64.1, 63.0, 61.0, and 58.9 G ± 0.5 G at 5, 15, 25, and 35°C, respectively.

The results of Fig. 3 show that the distribution of all the spin labels between the two environments is essentially independent of temperature. In these experiments the exchange rate between the boundary and the bilayer is slow on the ESR time scale (i.e., for these data the exchange rate is less than 10^7–10^8 sec^{-1}). At sufficiently elevated temperatures, this exchange rate may become detectable by ESR although we do not observe this up to 35°C. Recent NMR experiments on other proteins are interpreted as showing that the exchange rate for a neutral lipid is fast on the NMR time scale (22, 23). These results are compatible, since the ESR time scale is several orders of magnitude shorter than that of NMR.

Salt Suppression of the Charge Selectivity. Fig. 4 shows the effects of added salt on the ESR spectra of the methyl phosphate and quaternary amine labels. The difference between the labels was almost completely suppressed at high-salt concentration (2 M LiCl). Addition of 0.4 M NaCl or 0.4 M sodium phosphate (pH 7.3) had a much smaller effect.

The quantitative results with pairwise subtraction are shown in Fig. 5. In low-salt medium, the differences between the two labels are large. At high-salt concentrations, the fraction of bound label converges toward 0.48 ± 0.04 for both labels. At this point the binding is nonselective and the spin labels likely reflect the average distribution of the membrane lipid between the two environments. Assuming this to be the case, the estimated amount of boundary lipid is on the order of 0.15 mg/mg of protein or 61 mol of boundary lipid per mol of Na,K-ATPase, assuming the $\alpha_2\beta_2$ dimer as the functional unit with a molecular weight of ≈314,000 (unpublished data). The effect of salt was independent of temperature and could be completely reversed when the labeling was done after treatment of the Na,K-ATPase sample in 2 M LiCl followed by dilution into the low-salt buffer.

DISCUSSION

These results demonstrate that the Na,K-ATPase protein influences the lipid composition at the lipid–protein interface, tending to segregate negatively charged species. The lipids are in equilibrium between the protein-bound sites and the fluid bilayer. This equilibrium shifts, depending on the charge of the

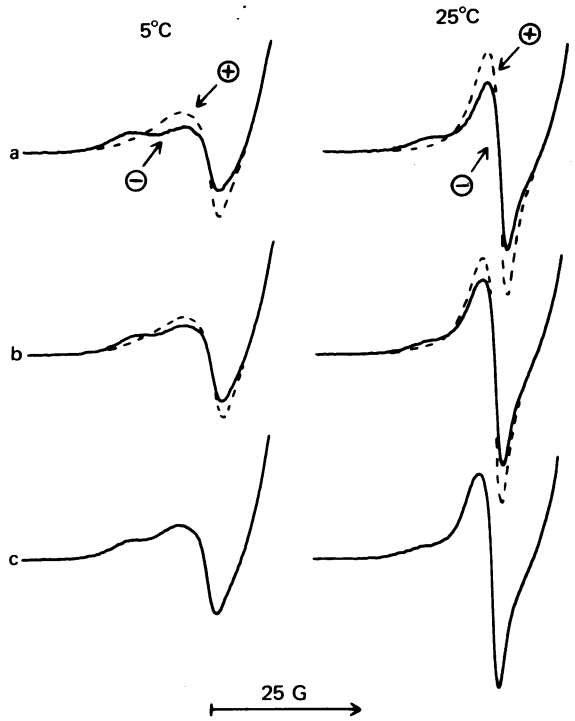

FIG. 4. ESR spectral changes in the low-field region observed with the addition of salt to Na,K-ATPase samples in the low-ionic strength buffer. Solid lines, negatively charged methyl phosphate lipid spin label; dashed lines, positively charged quaternary amine spin label. Spectra: a, no added salt; b, 0.4 M NaCl; c, 2 M LiCl. All spectra are scaled to the same integrated intensity to reflect the same number of spins. In high-salt medium, the two lines superimpose to within the width of the pen line.

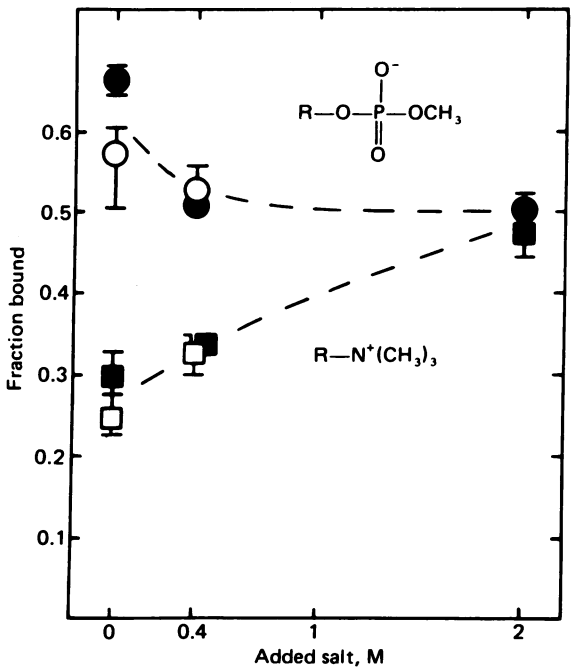

FIG. 5. The fraction of two lipid spin labels with oppositely charged head groups associated with the Na,K-ATPase as a function of salt concentration. Circles, label with negatively charged methyl phosphate head group (1); squares, label with positively charged quaternary amine head group (4). Two types of pairwise subtractions were used: solid symbols indicate pairwise subtraction of two spectra of one spin label at different salt concentrations; open symbols are points obtained by pairwise subtraction of spectra of the two labels at the *same* salt concentration. Within experimental error, the salt effect was independent of temperature over the range examined (5–35°C), and the points plotted are means of two to four pairs of spectra at different temperatures; the vertical bars show the range of values. The initial low-salt buffer was 0.25 M sucrose/1 mM Tris/1 mM EDTA, pH 7.3. The added salt was either NaCl (0.4 M) or LiCl (2 M). LiCl was chosen for the high-salt concentration for technical reasons (to adjust the density of the medium for sedimenting the Na,K-ATPase).

polar head group: the average fraction bound decreases from ≈0.57 (negative charge) through ≈0.35 (neutral) to ≈0.25 (positive charge). The equilibrium constant for the exchange between two environments is proportional to the ratio of bound to bilayer components (19). These ratios are approximately 1.3, 0.54, and 0.33 for the negative, neutral, and positive labels, respectively. This indicates that the average affinity of the protein for the negatively charged label is about 4 times larger than it is for the analogous positive species and about 2.5 times larger than it is for the neutral species when the lipid background is a mixture of native lipids.

The use of the newly synthesized phosphate spin labels eliminates any problems of pH- or salt-dependent charge that may occur when the traditional fatty acid spin labels are used (24). A phosphate diester (1) carries a constant negative charge over a wide pH range, and the phosphate triester (2) is neutral. All lipid labels used are identical except for the head groups, so that any effects caused by the perturbation of the spin label are constant in all experiments and cannot be responsible for the differences observed. The new method of data analysis used here avoids the experimental problem of varying the lipid-to-protein ratio in quantitating the shift in equilibrium associated with the charge. The results are in agreement with those of the single-component method of subtracting a presumptive bound component approximated by the lipid-deficient samples.

By convention, the lipid spin labels associated with the protein are referred to as immobilized or bound, because the spectral line shape resembles a nitroxide powder pattern. However, there is clearly some motion present on the ESR time scale as evidenced by the magnitudes and the temperature dependence of the maximal splitting ($2A_{max}$). This quantity is a function of the frequency and amplitude of motion. To obtain a general idea of the degree of motion of the bound label, it is useful to use the simple wobble model based on rapid random motion in a cone of half-angle γ (25).¶ From the $2A_{max}$ data (see *Results*) the calculated values of γ are 25°, 28°, 32°, and 36° at 5, 15, 25, and 35°C, respectively. The values of γ for the bilayer are much larger, in the range of 60–75°, although this model is probably less applicable in characterizing the very high amplitude motion because of the range of the frequencies present. Qualitatively, *on the ESR time scale*, the motion of the lipid in the bilayer is large and the motion is quite restricted when the lipid is in contact with the protein.

The spectral analysis shows that the binding equilibria are very nearly independent of temperature over the physiologically important range 5–35°C. Although there may be a small decrease in the binding of the negatively charged lipid label, it is within experimental error. From visual inspection of Fig. 1, it would be easy to reach the erroneous conclusion that the amount of bound spin label decreases substantially with increasing temperature. This visual impression is largely due to the increasing motion of the bilayer components with increasing temperature. With more rapid motion, the lines narrow and the amplitude increases, which tends to obscure the bound component. (A similar mistake in interpretation can occur when the lipid-to-protein ratio is increased so that the increased bilayer component gives the impression that the amount of bound lipid is decreasing.)

There are few data in the literature on the temperature dependence of boundary lipid. Two recent spin-labeling studies reached opposite conclusions regarding the temperature dependence of the boundary lipid of rhodopsin (16, 17). The first paper (16) involved reconstituted covalently labeled rhodopsin and concluded that most of the lipid at the protein surface becomes fluid (i.e., indistinguishable from the bilayer) at higher temperatures, although the extent of this effect is influenced by the lipid-to-protein ratio. One assumption made in this study was that the immobilized line shape was independent of temperature—i.e., the same immobilized reference spectrum was used for subtractions at all temperatures. We tested the effect of this assumption with our data and found that it artificially decreased the estimate of the lipid bound to the Na,K-ATPase (Fig. 3 *Inset*). Therefore, it may be necessary to reevaluate some of the interpretation in this rhodopsin study. The second study (17) involved rod outer segment membranes in which the broad spectral component is visible at all temperatures tested, and it was recognized that the $2A_{max}$ values were temperature-dependent. Although determination of $2A_{max}$ from the spectra shown in this paper is difficult, two different single-component subtraction methods gave values that showed little or no temperature dependence of the fraction of the bound component. Our data are consistent with these latter observations.

¶ Values of γ were calculated from equations 28 and 32 of ref. 25. The principal values of the A tensor of proxyl nitroxides have not been determined. Therefore, the principal values of the closely related nitroxide 2-doxylpropane (21) were used and scaled to the reference value of $2A_{max}$ = 69.2 G measured for a 14-proxylstearic acid derivative in cytochrome oxidase at 77 K. The resulting scaling factor corrects for the polarity of the binding site and is consistent with the known isotropic hyperfine coupling constants of a proxyl spin label (26).

The differences between the binding of the different labels must be due to either the head group charge or geometry or both. The experiments involving increasing salt concentration demonstrate that the main effect is due to the charge on the polar head group. The screening effect of high salt concentration (2 M LiCl) suppresses the charge discrimination and the two charged labels converge in behavior until they are nearly identical. The charge preference is substantial even well above physiological salt concentrations (i.e., up to 0.4 M NaCl). Membrane protein preferences for acidic lipids have been reported in native specialized bacterial membranes (15, 18), suggesting that this phenomenon may have some generality.

The charge selectivity is probably due to positive amino acid side chains at the lamellar interface. Recent sequence data on glycophorin (27), histocompatibility antigens HLA-A2 and HLA-B7 (28), the coat protein of the filamentous phage fd (29, 30), and one of the smallest polypeptides of cytochrome oxidiase (31) show the presence of several arginines and lysines at the cytoplasmic end of a hydrophobic stretch at the carboxy termini of these polypeptides. Although the three-dimensional structures of these proteins are unknown, the sequencing data provide evidence that there are basic amino acids in the region where some of these polypeptides emerge from the lipid bilayer.

In the Na,K-ATPase, the charge selectivity of binding we observed is similar to the trend in efficiency of amphiphiles for the reactivation of the enzyme (2). This suggests that activation results from direct binding of some of the acidic lipids to sites on the protein. These ESR data provide the first experimental evidence that such direct binding does occur.

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