The interaction of divalent cations with (Na,K)-ATPase

A LIPID-BOUND FLUORESCENCE PROBE STUDY*

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The lipid-bound fluorescence probes dansyl phosphatidylethanolamine (DPE) and 12-(9-anthroyl)stearate (12-AS) have been incorporated into purified lamb kidney (Na,K)-ATPase and into the lipids extracted from (Na,K)-ATPase. Addition of 5 mM MgCl₂ or 1 mM CaCl₂ caused a 20% enhancement in the fluorescence of DPE incorporated into (Na,K)-ATPase (DPE-(Na,K)-ATPase) and a 50% enhancement in the fluorescence of DPE incorporated into extracted lipids (DPE-lipids). Monovalent cations lowered the enhancement in DPE-(Na,K)-ATPase fluorescence caused by divalent cations. The binding constants of each divalent cation as determined by the divalent cation-induced DPE fluorescence enhancement are: K₀ (DPE-(Na,K)-ATPase) Mg²⁺ = 0.95 mM; K₀ (DPE-lipids) Mg²⁺ = 0.83 mM; K₀ (DPE-(Na,K)-ATPase) Ca²⁺ = 0.115 mM; K₀ (DPE-lipids) Ca²⁺ = 0.185 mM. These results indicate that cations Mg²⁺, Ca²⁺, Na⁺, and K⁺ interact primarily with the polar head groups of the phospholipids. An efficient fluorescence energy transfer was observed from the tryptophan residues of (Na,K)-ATPase to DPE or to 12-AS located in the lipid bilayer. This energy transfer provides a convenient means of monitoring protein-lipid interactions in (Na,K)-ATPase. The efficiency of energy transfer was higher for (Na,K)-ATPase labeled at 4°C (below T₁) compared to (Na,K)-ATPase labeled at 37°C (above T₁), indicating two domains of lipids present in (Na,K)-ATPase membranes.

The membrane-bound (Na⁺ and K⁺)-dependent adenosine triphosphatase (Na,K)-ATPase, E.C. 3.6.1.3., is the enzymatic component of the sodium pump in eukaryotic cells (1). Magnesium and to a lesser extent Mn⁺⁺, Co⁺⁺, Fe⁺⁺, and Ni⁺⁺ activate the enzyme, while Ca⁺⁺ and many heavy metals inhibit (Na,K)-ATPase (2, 3). It has been suggested that (Na,K)-ATPase undergoes a large conformational change upon the binding of Mg⁺⁺ (4). This conformational change in (Na,K)-ATPase may be propagated to the boundary lipids or to the bulk lipids, causing changes in the affinity of the lipids for the different ions. The inhibition of (Na,K)-ATPase by Ca⁺⁺ may be due, in part, to a reduction in the apparent affinity of the lipids of the enzyme for Mg⁺⁺ and Na⁺⁺ (3). It is well accepted that lipids are essential for the enzymatic function of (Na,K)-ATPase, although reports of the role and the specificity of this lipid requirement are often conflicting (1, 5).

Numerous investigators have used inactivation by lipases, or detergent or organic solvent extraction procedures followed by reactivation with specific phospholipids for their studies (6). An alternative method for investigating the role of lipids in the (Na,K)-ATPase is the use of lipid-bound fluorescence probes (7, 8).

Dansyl phosphatidylethanolamine (DPE) and 12-(9-anthroyl)stearate (12-AS) are useful fluorescence probes for the investigation of the role of lipids in the membrane because the fluorescent moiety is covalently attached to a phospholipid or to a fatty acid. The dansyl group in DPE is located in the glycerol region and is, therefore, able to monitor the structural changes in the polar head groups. The anthroyl group in 12-AS is located in the hydrophobic region and reports changes in the fluidity of the membrane. Cation-induced structural changes in lipid vesicles, dispersions and micelles have been detected using DPE (9) and dansyl phosphatidylserine (10). Using N-phenyl-2-naphthalamine and 12-AS, the lipid mobility of membranes containing (Na,K)-ATPase has been related to the phase transition and thermal activation of the enzyme (11). No attempt has yet been made to investigate possible cation-induced structural changes in the (Na,K)-ATPase membrane.

To investigate the effects of divalent cations on the interaction of (Na,K)-ATPase protein with membrane lipids, DPE and 12-AS were incorporated into a purified (Na,K)-ATPase and into lipids extracted from the (Na,K)-ATPase.

MATERIALS AND METHODS

(Na,K)-ATPase was purified from lamb kidney outer medulla as described by Lane et al. (12). The glycerol precipitated fraction was dialyzed against 10 mM Tris-Cl, pH 7.4, to remove residual detergent and EDTA. The specific activity of (Na,K)-ATPase was 900 to 1,000 pmol of P₃/μg/h as measured at 37°C using a spectrophotometric-coupled enzyme assay described previously (12). Lipids were extracted from lamb kidney NaI fraction essentially as described by Folch et al. (13) and stored in chloroform:methanol (2:1) at -20°C in a nitrogen atmosphere until used. The (Na,K)-ATPase fraction contained 1.1 μmol of lipid phosphorus and 0.3 μmol of cholesteryl/mg of protein, as determined by the method of Bartlett (14) and Zlatkis et al. (15), respectively. The relative quantities of the individual phospholipid classes, determined as phosphorous (14), following one-dimensional thin layer chromatography according to Skipski et al. (16) are: phosphatidylcholine = 44%, phosphatidylethanolamine = 36%, and phosphatidylyserine plus phosphatidylinositol = 17%. Because one dimen-

* This work was supported in part by United States Public Health Service Grants PO1 HL 22619-01 (Project I-E), HL 22039-02, HL 22109, and T32 HL 07382-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡ Postdoctoral trainee.§ Fellow of the Muscular Dystrophy Association.† To whom correspondence regarding this paper should be addressed.

The abbreviations used are: (Na,K)-ATPase, (Na⁺,K⁺)-dependent adenosine triphosphatase; DPE, dansyl phosphatidylethanolamine; dansyl, 5-dimethylaminonapthalene-1-sulfonyl; 12-AS, 12-(9-anthroyl)stearate; SDS, sodium dodecyl sulfate; DPE-(Na,K)-ATPase, DPE incorporated into (Na⁺,K⁺)-ATPase; DPE-lipids, DPE incorporated into the extracted lipids; 12-AS-(Na,K)-ATPase, 12-AS incorporated into (Na⁺,K⁺)-ATPase.
sional thin layer chromatography of the chloroform:methanol extracted phospholipids revealed that the phospholipid complements of the NaI-microsomes and glycerol precipitated fractions of (Na,K)-ATPase were essentially the same, the NaI microsome fraction was used as a source for extracted lipids. Lipid vesicles were prepared by drying the lipids onto the wall of a glass test tube with a gentle stream of nitrogen, followed by the addition of 10 mM Tris-Cl buffer, pH 7.4, to form a suspension, and solubilization by the addition of 3% sodium cholate. The sodium cholate was then removed by dialysis against 10 mM Tris-Cl buffer, pH 7.4 at 4°C. Lipid phosphate was determined according to Bartlett (14); protein by the method of Lowry et al. (17) with bovine serum albumin as the standard. Sarcoplasmic reticulum from rabbit back muscle was prepared as described previously (18).

Both DPE and 12-AS were obtained from Molecular Probes, DPE as a chloroform:methanol solution. In addition, dansyl phosphorylethanolamine was synthesized by the method of Waggoner and Stryer (19) and stored in chloroform in the dark under nitrogen at -20°C. The ethanolic solutions of the probes were added to (Na,K)-ATPase or to the lipids extracted from the NaI-microsome fraction (referred to as extracted lipids hereafter) such that the concentration of ethanol was always less than 2%. The DPE which was synthesized in our laboratory and stored in chloroform, was coated onto the wall of a test tube by evaporation with a gentle stream of nitrogen. Extracted lipids or (Na,K)-ATPase were added to the tube. The probes were incorporated into the membrane by mixing with a vortex for 10 sec, followed by sonication in a Cole-Parmer bath type sonicator at 4°C or at 37°C for 5 min. The ratio of probe to lipid was approximately 1.50 such that 25 nmol of DPE were added to 1.1 nmol phospholipid/mg. The specific activity of (Na,K)-ATPase was not altered by the sonication. Unincorporated DPE was removed by repeated washes and centrifugation. The labeled enzyme could be stored at 3°C (10 mg/ml) for several weeks without loss of activity. The MgCl₂ and CaCl₂ solutions were adjusted to pH 7.4 by the addition of Tris base, and the pH change due to the addition of the divalent cations to the labeled enzyme was less than 0.1 unit.

(Na,K)-ATPase labeled with DPE dissolved in chloroform reproducibly showed a greater divalent cation-induced fluorescence enhancement and a lower efficiency energy transfer than did (Na,K)-ATPase labeled with DPE dissolved in ethanol. For this reason, (Na,K)-ATPase was labeled with DPE dissolved in chloroform for the cation induced fluorescence enhancement studies and with DPE dissolved in ethanol for the energy transfer studies. The DPE obtained from both sources migrated as a single spot with identical Rₑ values of 0.6 on one-dimensional thin layer chromatography (Merck, Silica Gel 60, 0.25 mm) with chloroform:methanol:water (70:30:4). Judging from the absorption and fluorescence spectra of the labeled (Na,K)-ATPase and extracted lipids, equal quantities of probe were incorporated regardless of the source. The reasons for these differences are obscure at the present time.

Emission and excitation spectra were obtained at 24°C using a Perkin-Elmer MPF44A fluorescence spectrometer in the ratio mode and excitation mode, respectively, at a slit width of 4 nm for both. The concentrations of DPE were determined by absorption spectroscopy assuming an ε₅₄₀ of 3,400 M⁻¹ cm⁻¹ for the dansyl fluorescence moiety (20). The excitation wavelength was 340 nm for all emission spectra. For excitation spectra, the excitation wavelength was set at 480 nm so that the scattering peak at 240 nm would not interfere with the protein excitation.

RESULTS AND DISCUSSION

Dansyl phosphatidylethanolamine was incorporated into (Na,K)-ATPase (DPE-(Na,K)-ATPase) and into the extracted lipids (DPE-lipids). The wavelength of the emission maximum (λₘ₇₃₃) of the DPE-(Na,K)-ATPase occurred at 512 nm (Fig. 1, lower trace), whereas the λₘ₇₃₃ of the DPE-lipids occurred at a longer wavelength, 517 nm. This blue-shift of DPE fluorescence from 517 nm in the extracted lipids to 512 nm in the (Na,K)-ATPase indicates that the probe is in a more rigid and/or hydrophobic environment in the (Na,K)-ATPase than it is in the environment of the extracted lipids. We favor an interpretation that this environment of the probe in (Na,K)-ATPase is more rigid than that in the extracted lipids over the alternate interpretation that there are differences in hydrophobicity, since the presence of a protein in the lipid matrix is known to increase the rigidity of membranes (8, 21).

The addition of 5 mM MgCl₂ enhanced the fluorescence intensity of DPE-(Na,K)-ATPase by 20% and blue-shifted the λₘ₇₃₃ from 512 to 508 nm (Fig. 1). The fluorescence enhancement of DPE-lipids upon the addition of 5 mM MgCl₂ was 50% and the blue shift in the λₘ₇₃₃ was from 517 to 510 nm. The addition of 1 mM CaCl₂ also caused a 20% enhancement in DPE-(Na,K)-ATPase and a 50% enhancement in DPE-lipids with similar shifts in λₘ₇₃₃. It is well known that cations bind primarily to the polar head groups of the membrane lipids (9, 22). The enhancement of fluorescence intensity and blue-shifts in the λₘ₇₃₃ of DPE-(Na,K)-ATPase and DPE-lipids probably reflect a divalent cation-induced increase in the rigidity of the membrane, since these phenomena have been observed in other membrane systems (23, 24). The 50% enhancement and 7 nm blue shift of DPE fluorescence in DPE-lipids presumably reflect a maximum increase in the rigidity of the membrane due to the binding of divalent cations to the lipids. As suggested above, the presence of protein ((Na,K)-ATPase in this case) in the lipid matrix itself increases the rigidity of the membrane prior to the addition of divalent cations. Consequently, although binding of the divalent cations to both DPE-(Na,K)-ATPase and DPE-lipids increase their rigidity, the extent of the increase in the rigidity is smaller in (Na,K)-ATPase, due to the initial higher rigidity. The extent of the blue-shift induced by divalent cations (512 to 508 nm in DPE-(Na,K)-ATPase, 517 to 510 nm in DPE-lipids) may also reflect this difference in initial rigidity.

Incorporation of DPE into sarcoplasmic reticulum vesicles containing Ca²⁺-ATPase caused fluorescence changes similar to those observed with (Na,K)-ATPase upon addition of Mg²⁺ or Ca²⁺ (data not shown). These fluorescence changes suggest that the fluorescence enhancement is due to the binding of the divalent cations to lipids rather than due to a specific interaction with the protein components.

The divalent cation-induced enhancement in DPE-(Na,K)-ATPase fluorescence was reduced by the subsequent addition of monovalent cations. The addition of 5 mM MgCl₂, followed by the addition of 100 mM NaCl reduced the divalent cation-induced enhancement and the λₘ₇₃₃ of the DPE-lipids was blue-shifted from 517 to 510 nm (Fig. 1, upper trace).
induced fluorescence enhancement to only about 5%. Addition of monovalent cation (100 mM Na⁺) alone added to DPE-(Na,K)-ATPase resulted in a 5% decrease in the intensity of fluorescence. The subsequent addition of divalent cation (5 mM Mg²⁺) increased the intensity of the DPE fluorescence only to the level observed in the absence of any cations. Similar results were obtained with the addition of Mg²⁺ and K⁺, Ca²⁺ and Na⁺, and Ca²⁺ and K⁺. The addition of monovalent cations to lipids is known to increase the fluidity of the bilayer structure at a particular temperature, in contrast to the more ordered structure obtained by the addition of divalent cations. High concentrations of monovalent cations are also known to displace divalent cations from their polar head group binding sites (25, 26). The monovalent cation-induced decrease in DPE-(Na,K)-ATPase fluorescence is consistent with this concept, and indicates that the divalent cations bind primarily to the polar head groups of the (Na,K)-ATPase phospholipids and can be displaced by high concentrations of monovalent cations.

The binding of Ca²⁺ and Mg²⁺ to DPE-(Na,K)-ATPase and to DPE-lipids was monitored by the increase in the DPE fluorescence. The fluorescence increases saturated at 1 mM Ca²⁺ or 5 mM Mg²⁺ for both DPE-(Na,K)-ATPase and DPE-lipids. The increase in fluorescence of DPE-lipids, which occurred with successive additions of Ca²⁺ (0 to 1 mM), is shown in Fig. 2A. At 1 mM Ca²⁺, a 50% enhancement and a 7 nm blue shift in $\lambda_{max}$ of DPE-lipids were observed. Subsequent addition of 2 mM EDTA reduced the fluorescence of DPE-lipids slightly below the control level, indicating the presence of endogenous Ca²⁺ (less than 50 μM) in the reaction medium. Titration of the fluorescence increase in DPE-(Na,K)-ATPase and DPE-lipids that occur with the addition of Mg²⁺ and Ca²⁺ were analyzed by double reciprocal plots (Fig. 2B). The binding constants obtained are $K_D$ (DPE-(Na,K)-ATPase) Mg²⁺ = 0.95 mM; $K_D$ (DPE-lipids) Mg²⁺ = 0.83 mM; $K_D$ (DPE-(Na,K)-ATPase) Ca²⁺ = 0.115 mM, $K_D$ (DPE-lipids) Ca²⁺ = 0.185 mM. These values are in close agreement with those reported by Gervais et al. (3) determined by direct "Ca binding ($K_D$ (Na,K)-ATPase) Ca²⁺ = 0.10 mM; $K_D$ (lipids) Ca²⁺ = 0.15 mM) and by Kuriki et al. (4) by calorimetric studies ($K_D$ (Na,K)-ATPase) Mg²⁺ = 0.83 mM). The binding constants obtained for Ca²⁺ binding to phosphatidylserine by several methods (26) also agree with the binding constant of Ca²⁺ to (Na,K)-ATPase lipids. The double reciprocal plots obtained by plotting the enhancement of DPE fluorescence against the cation concentration are linear, indicating a single class of binding sites for the divalent cations. The binding constants of these divalent cations for DPE-(Na,K)-ATPase and DPE-lipids are similar, suggesting that the Ca²⁺ and Mg²⁺ binding sites in both systems are the polar head groups of the phospholipids. Divalent cation binding to the phospholipid head groups of the (Na,K)-ATPase is known to displace divalent cations from their polar head group binding sites (25, 26). The monovalent cation-induced decrease in DPE-(Na,K)-ATPase fluorescence is consistent with this concept, and indicates that the divalent cations bind primarily to the polar head groups of the (Na,K)-ATPase phospholipids and can be displaced by high concentrations of monovalent cations.

It has been proposed that the phospholipids of the cell membrane serve as a Ca²⁺ sink, and therefore may be involved in the regulation of contractin in the myocardium and perhaps in the mechanism of the inotropic effect of digitalis (27). Since Ca²⁺ binding to and removal from the phospholipid head groups of the (Na,K)-ATPase membrane are easily monitored with DPE fluorescence, this technique should prove very useful in relating these Ca²⁺-membrane interactions to inotropy.

The fluorescent-labeled fatty acid 12-AS was incorporated into the bilayer of (Na,K)-ATPase and into the extracted lipids in an effort to detect cation-induced structural changes in the hydrophobic region of the bilayer. No fluorescence changes in 12-AS were observed with the addition of either divalent (Mg²⁺, Ca²⁺) or monovalent (Na⁺, K⁺) cations. These observations suggest that the structural changes occurring with the binding of cations to DPE-(Na,K)-ATPase or DPE-lipids are either less dramatic in this region of the bilayer or that the anthroyl moiety of 12-AS is less sensitive to these changes than the dansyl moiety of DPE and is, therefore, unable to detect such changes.

Because of their excellent spectral overlap and high quantum efficiencies, tryptophan and the dansyl fluorophore have often been used as a donor-acceptor pair in Forster resonance energy transfer studies (28). The absorption spectrum of DPE-(Na,K)-ATPase shows the characteristic DPE "shoulder" at 340 nm compared to the native (Na,K)-ATPase (Curves A and B, Fig. 3). The emission spectrum of the tryptophan of (Na,K)-ATPase (Curve C) exhibits a $\lambda_{max}$ at 335 nm which overlaps with the excitation spectra of DPE in ethanol (Curve D) and DPE-(Na,K)-ATPase (Curve E) in the 340 nm range. Thus, in this system, tryptophan should be an efficient energy donor to DPE if the proximity and orientation requirements are met (28).

An efficient energy transfer from the tryptophan, and possibly the tyrosine, residues of (Na,K)-ATPase to DPE incorporated into the lipid matrix was observed. This is shown by the excitation spectra (Curve E, Figs. 3 and 4). A large excitation peak at 283 nm is observed in DPE-(Na,K)-ATPase but not in DPE in ethanol (Curve D, Fig. 3) or in DPE-lipids (Fig. 4, bottom trace). This excitation peak at 283 nm must result from the transfer of energy from the tryptophan and/or tyrosine residues of (Na,K)-ATPase to DPE, since it corresponds with the absorption spectrum of the protein (Curve A of Fig. 3). The tyrosine residues of the protein would not be
The gel + liquid crystalline to liquid crystalline phase transition, \( T_h \), (29) is reported to be 17°C for (Na,K)-ATPase isolated from eel electroplax (30) and 22°C for enzyme isolated from sheep kidney (11). The purified (Na,K)-ATPase used in this study had a \( T_h \) of 18°C as measured by the fluorescent probe diphenyl hexatriene. The gel to gel + liquid crystalline phase transition (\( T_l \)) is reported to occur at 8°C (30). Dansyl phosphatidylethanolamine was incorporated into (Na,K)-ATPase at 37°C (above \( T_h \)) and both DPE and 12-AS were incorporated at 4°C (below \( T_l \)). The fluorescence energy transfer efficiency for each labeled (Na,K)-ATPase was determined by the equation:

\[
T = \frac{F_{283} - e_{283}^{\text{a}} e_{340}^{\text{a}}}{F_{283} - e_{283}^{\text{a}} e_{345}^{\text{a}}}
\]

where \( e^a \) and \( e^d \) are the extinction coefficients of the probe (DPE) acceptor and protein donor, respectively, and \( F_{283} \) and \( F_{345} \) are the fluorescence intensities at the respective wavelengths of the excitation spectra (\( \lambda_{ex} = 480 \) nm). The values of the extinction coefficients were 5180, 850, and 3400 \( \text{m}^{-1} \text{cm}^{-1} \) for \( e_{283}^{\text{a}}, e_{283}^{\text{a}} \), and \( e_{340}^{\text{a}} \) (31). Similarly, the energy transfer efficiency for 12-AS-(Na,K)-ATPase was calculated by \( F_{283}, F_{345} \) intensities monitored at 435 nm. The values of the extinction coefficient of the acceptor \( e_{285}^{\text{a}} \) and \( e_{340}^{\text{a}} \) for 12-AS were 100 and 5290 \( \text{m}^{-1} \text{cm}^{-1} \), respectively. Using these values, the efficiency of energy transfer were: 0.38 for DPE-(Na,K)-ATPase and 0.28 for 12-AS-(Na,K)-ATPase labeled at 4°C and 0.083 for DPE-(Na,K)-ATPase labeled at 37°C. The efficiencies of energy transfer were 3- to 4-fold higher for DPE labeling at 4°C compared with that at 37°C. Further, the divalent cation-induced fluorescence enhancement of DPE-(Na,K)-ATPase labeled at 37°C was 20%, while that of DPE-(Na,K)-ATPase labeled at 4°C was about 8%.

Above the \( T_h \) the bulk lipids are in a more fluid state compared to the boundary lipids. Below \( T_l \) the bulk lipids are “frozen” in the gel state and are less fluid than the boundary lipids. Under these conditions, the protein and surrounding boundary lipids are excluded from the more rigid bulk lipid domain (29). The different efficiencies of energy transfer and expected to contribute substantially to this excitation peak because of the poor spectral overlap of their emission with DPE absorption and because of their lower quantum efficiency. This energy transfer (as monitored by the 283 nm excitation peak) was completely abolished by 1% SDS. This observation of energy transfer from the tryptophans of (Na,K)-ATPase to DPE in the lipid matrix indicates that some fraction of the DPE molecules must be within 70 Å of the protein. These DPE molecules may be removed by detergents such as SDS, deoxycholate and Triton X-100, which are known to delipidate and inactivate the (Na,K)-ATPase (5), resulting in the complete disruption of the energy transfer. This energy transfer, therefore, can be used to monitor structural changes which alter protein lipid interactions.

An efficient fluorescence energy transfer from tryptophan residues of (Na,K)-ATPase to the 12-AS in the lipid matrix was also observed as shown in the excitation spectrum of Fig. 5. The presence of the 283 nm excitation peak (Fig. 5) is again indicative of this energy transfer. This peak is absent in the 12-AS-lipids and is abolished when the (Na,K)-ATPase is delipidated with SDS.

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different enhancements of DPE-(Na,K)-ATPase fluorescence are consistent with the concept that above $T_s$, the DPE molecules are randomly incorporated into the fluid lipid matrix, and that a small fraction of them are within the energy transfer distance. Below $T_l$, due to a relatively less rigid environment of the boundary lipids compared to the bulk lipids, DPE is presumably preferentially incorporated into the boundary lipid domain giving rise to a highly efficient energy transfer. These experiments suggest the presence of two domains of (Na,K)-ATPase lipids. One is the bulk lipids; DPE incorporated into this domain exhibited a 20% enhancement with divalent cation binding and a low (0.083) efficiency of energy transfer. The other domain is the boundary lipid. DPE incorporated into these lipids showed a smaller divalent cation-induced enhancement and a higher (0.38) efficiency of energy transfer. As the boundary lipids are relatively immobile compared to the bulk lipids, probe molecules located in the boundary lipid domain may remain in the proximity of the protein even at higher temperatures. Also, some of the probe molecules near the protein may be oriented more favorably for energy transfer perhaps because of a specific association with the catalytic or glycoprotein subunit. The binding of divalent cations to the boundary lipids may cause a smaller increase in their rigidity and, therefore, a smaller divalent cation-induced increase in the fluorescence intensity of DPE incorporated into this domain.

The efficient energy transfer that occurs between the tryptophan residues of the (Na,K)-ATPase and the fluorescence acceptor molecules (DPE and 12-AS), that are incorporated preferentially into the boundary lipids as described above, is a convenient means of monitoring protein-lipid interactions within a distance of 70 Å. This technique, therefore, offers a new approach for testing the hypothesis (3, 27, 32) that conformational changes in the protein moiety of (Na,K)-ATPase are transmitted to adjacent phospholipids.

**Acknowledgments**—We are grateful to Ms. Gwen Kraft for her expert artwork and Ms. Linda Miner for assistance in the preparation of the manuscript.

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