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Modulation of (Na^+,K^+)-ATPase Activity by the Lipid Bilayer Examined with Dansylated Phosphatidylserine†

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ABSTRACT: The fluorescent probe 8-(dimethylamino)naphthalene-1-sulfonylphosphatidylserine (Dns-PS) was incorporated into purified lamb kidney Na^+- and K^+-stimulated adenosinetriphosphatase (EC 3.6.1.3) [(Na^+,K^+)-ATPase] by using a purified phospholipid exchange protein. Phospholipase C was used to reduce phospholipid content. Up to 40% of the phospholipid could be hydrolyzed with only 10% inhibition of the (Na^+,K^+)-ATPase, but when 67% of the phospholipid was hydrolyzed, the enzyme was inhibited 53%. To examine the effect of protein on the phospholipid bilayer, the fluorescent parameters of the probe incorporated into the enzyme preparation were contrasted with the same parameters for the probe incorporated into the total lipid extract of the preparation. The polarization of fluorescence of the probe in the lipid extract was 0.118 while in the enzyme preparation it was 0.218. This reflected a decrease in fluidity of the glycerol region of the phospholipid bilayer which was mediated by the protein. This effect increased as the phospholipid content of the (Na^+,K^+)-ATPase preparation was reduced so that with maximal phospholipid reduction the polarization of fluorescence was 0.262. The protein caused a decrease in the transition temperature from gel to fluid states of the bilayer detected by polarization of the probe. The midpoint temperature transition of the enzyme preparation decreased from 33 °C when all phospholipids were present to 20 °C when 67% of the phospholipids were hydrolyzed. This decrease was not observed for the lipid extract of these samples. A direct correlation between the (Na^+,K^+)-ATPase specific activity and the polarization of fluorescence of Dns-PS was found. The reduction in phospholipid content did not affect the steady-state level of phosphorylation of the enzyme by ATP but did affect the rate of dephosphorylation which would require conformational changes of the enzymes. The data showed that the fluidity of the phospholipid bilayer can modulate the activity of the (Na^+,K^+)-ATPase.

The Na^+- and K^+-stimulated adenosinetriphosphatase, (Na^+,K^+)-ATPase[1] (ATP phosphohydrolase, EC 3.6.1.3), is an intrinsic membrane enzyme that couples the hydrolysis of ATP to the active transport of Na^+ and K^+ across biological membranes. This enzyme requires the presence of phospholipids for full activity since their removal will cause partial to complete loss of activity (Kimelberg & Papahadjopoulos, 1972; Jorgenson, 1982; Stahl, 1973a; Simpkin & Hokin, 1973). Partial recovery of activity has been achieved by reintroduction of phospholipids, but the results do not seem to clearly support a requirement for a specific phospholipid class (Jorgenson, 1982; Hokin & Hexum, 1972; Giraud et al., 1981; Ottolenghi, 1979).

Brotherus et al. (1980) suggested that negatively charged phospholipids preferentially associated with the (Na^+,K^+)-ATPase, on the basis of an electron spin resonance (ESR) study with spin-labeled phospholipids. In contrast, DePont et al. (1978), employing specific lipases, enzymatically converted all the phosphatidylserine in a (Na^+,K^+)-ATPase preparation to phosphatidylethanolamine with essentially no effect on (Na^+,K^+)-ATPase activity. These authors also enzymatically hydrolyzed all the phosphatidylinositol in the (Na^+,K^+)-ATPase sample, again without an effect on the enzyme activity. Hilden & Hokin (1976), using excess ex-

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[1] Abbreviations: (Na^+,K^+)-ATPase, Na^+- and K^+-stimulated adenosinetriphosphatase (EC 3.6.1.3); Dns-PS, 8-(dimethylamino)-naphthalene-1-sulfonylphosphatidylserine; PLEP, phospholipid exchange protein; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; SM, sphingomyelin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane (Trizma base); SET, 250 mM sucrose, 50 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid buffer, pH 7.4; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; T_m, midpoint temperature transition; PLP, phospholipid phosphorus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESR, electron spin resonance; Cl_3CCOOH, trichloroacetic acid.

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ogenous phospholipid and detergent to replace endogenous phospholipids, obtained an active (Na^+,K^+)-ATPase preparation containing only phosphatidylcholine, a neutral phospholipid. It is likely that the procedures used for lipid depletion or lipid insertion account for a portion of the discrepancies in these reports.

This study employed a nonspecific phospholipid exchange protein (PLEP) isolated from bovine liver which allowed the introduction of specific labeled phospholipids into biological membranes under mild physiological conditions. The PLEP is a low molecular weight soluble protein easily separated from the membranous (Na^+,K^+)-ATPase preparation. The fluorescent phospholipid 8-(dimethylamino)naphthalene-1-sulfonylphosphatidylserine (Dns-PS) was introduced by using PLEP. The properties of the lipid region of the preparation were monitored by measuring fluorescent parameters of the dansylated phospholipid.

An earlier report in which Dns-PS was used as a lipid probe within the (Na^+,K^+)-ATPase found essentially no correlation between enzymatic conformations and changes in the fluorescent properties of the probe (Muczynski & Stahl, 1983). The purpose of this study was to increase the proportion of the probe in the sample so that a higher proportion of Dns-PS might interact with the (Na^+,K^+)-ATPase at the protein–lipid interface. This was accomplished by decreasing the total phospholipid content by hydrolysis of PC and sphingomyelin (SM) with phospholipase C from *Clostridium welchii*. The phospholipase C hydrolyzed PC and SM but does not attack phosphatidylserine (Stahl, 1973b). This lipase treatment should increase the probability of having the probe in close proximity to the protein portion of the enzyme preparation.

Materials and Methods

The (Na^+,K^+)-ATPase enzyme was prepared from the inner medulla of lamb kidneys by the method described by Lane et al. (1979) and was stored on ice until used. Specific enzyme activities of between 1000 and 1300 μmol of P_i (mg of protein)$^{-1}$ h^{-1} were routinely obtained. The preparation was examined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and was found to be greater than 90% pure.

8-(Dimethylamino)naphthalene-1-sulfonylphosphatidylserine (Dns-PS) was synthesized by using the procedure of Waggoner & Stryer (1970) as modified by Harris & Stahl (1976). PS was purchased from the Supelco Corp., and dansyl chloride (DnsCl) was recrystallized from acetone prior to use. [^{3}H]DnsCl was purchased from New England Nuclear Corp. and was added so that the final product would have approximately 2000 cpm/nmol.

The nonspecific phospholipid exchange protein (PLEP) was purified as described by Crain & Zilversmit (1980) through the heat coagulation and concentration step. Specific activities of the PLEP prepared in our laboratory were between 400 and 560 nmol of phospholipid exchanged (mg of protein)$^{-1}$ h^{-1}, which was less than the activity reported by Crain & Zilversmit (1980) although mitochondria were used as acceptors in both cases. This purified protein gradually aggregated into insoluble material when stored at 4 °C, so it was used immediately after being isolated.

Phospholipid donor vessels were prepared by mixing phosphatidylcholine (PC) and Dns-PS in 2:1 chloroform/methanol in the desired proportions, usually 90% PC/10% Dns-PS, with 0.1 mol % butylated hydroxytoluene. The proportion of Dns-PS in the donor vessel suspension was maintained below 10% of the total lipid as greater proportions of this charged lipid cause nonspecific surface adherence without incorporation (Muczynski et al., 1983). The solution was taken to dryness

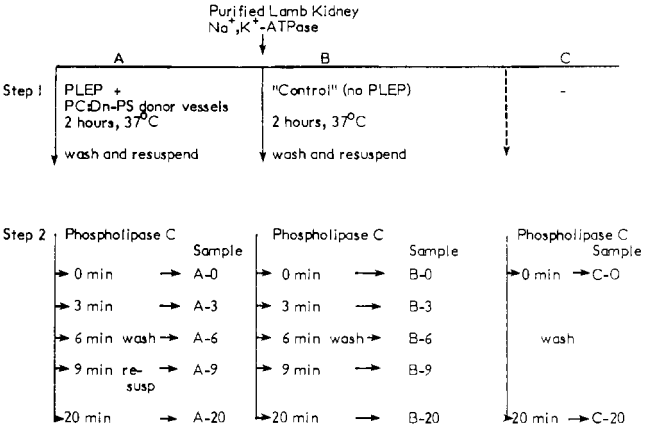

FIGURE 1: Incorporation of Dns-PS into purified (Na^+,K^+)-ATPase and reduction in phospholipid content. The experimental design to provide a series of (Na^+,K^+)-ATPase controls and samples with a fixed amount of fluorescent probe, Dns-PS, and decreased phospholipid content is shown. The fluorescent probe Dns-PS and PC were introduced in step 1 by using PLEP (column A). Control samples were treated identically but without PLEP (column B). The second step in this procedure was treatment with phospholipase *C* from *Clostridium welchii* for the indicated times. The initial (Na^+,K^+)-ATPase preparation was included as an additional control that had only the lipase treatment (column C).

with N_2. Buffer containing 250 mM sucrose, 50 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid, pH 7.4 (SET buffer), was added to produce 0.2 mM total phospholipid, and the material was vigorously vortexed. It was then sonicated with a Branson 200 probe sonifier (50% duty cycle, power setting 5) until clarified (usually 15–20 min). The material was then centrifuged for 30 min in a Spinco 40 rotor at 55000*g* to remove any larger aggregates.

The experimental scheme for this study is shown in Figure 1. In the first step, the exogenous phospholipids Dns-PS and PC were introduced into the (Na^+,K^+)-ATPase preparation by using a nonspecific phospholipid exchange protein (column A). A control sample without PLEP was run in parallel (column B). The Dns-PS-labeled sample (A) and the control sample (B) were treated with phospholipase C for varying times that would yield samples with varying phospholipid content. A third set of samples was prepared by treating the initial lamb kidney (Na^+,K^+)-ATPase only with phospholipase C (column C).

The incorporation of exogenous phospholipids into the (Na^+,K^+)-ATPase preparation was done at 37 °C for 60 min in a buffer containing 50 mM NaCl, 2.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 25 mM Tris-HCl, pH 7.4, and 5 mg of fatty acid poor bovine serum albumin/mL to maintain maximal (Na^+,K^+)-ATPase activity. The (Na^+,K^+)-ATPase concentration was 0.5 mg of protein/mL. The usual PLEP concentration was 0.4 mg of protein/mL, and the ratio of donor phospholipid to endogenous phospholipid was between 0.3:1 and 0.4:1. Controls were run concurrently without PLEP to measure nonspecific adhesion of the donor vessels to the (Na^+,K^+)-ATPase (Muczynski & Stahl, 1983). [^{14}C]Triolein, a nonexchangeable lipid (Crain & Zilversmit, 1980), was included as a measure of nonspecific adhesion. The reaction was terminated by cooling the solution on ice and then centrifuging it at 28 000 rpm in a Spinco 40 rotor for 30 min. The resulting pellet was washed twice with SET buffer containing 5 mg of BSA/mL to remove phospholipids adhered to the enzyme surface and then twice with SET buffer to remove the BSA. Following this procedure, the nonspecific surface adhesion of Dns-PS was less than 8% of the incorporated Dns-PS value, as measured by the radioactivity of the [^{3}H]Dns-PS in

Table I: Composition and Enzyme Activity of (Na^+,K^+)-ATPase Samples[a]

sample	time treated with phospholipase C (min)	PLP content (μmol of PLP/mg of protein)[b]	% control	% Dns-PS in total PLP	cholesterol content (mg/mg of protein)[c]	(Na^+,K^+)-ATPase sp act. [μmol (mg of protein)$^{-1}$ h^{-1}]	% control
			Dns-PS/PC (Na^+,K^+)-ATPase				
A-0	0	0.944	100	2.6	0.056	543 ± 14 (SD)	100
A-3	3	0.698	73.9	3.6	0.041	469	86.4 ± 3.4
A-6	6	0.575	60.9	4.3	0.041	492	90.6 ± 4.5
A-9	9	0.405	42.9	6.1	0.044	375	69.1 ± 2.4
A-20	20	0.305	32.3	8.2	0.043	255	46.9 ± 7.1
			Control (Na^+,K^+)-ATPase Treated As Described Above without PLEP				
B-0	0	0.905	100			672 ± 28	100
B-3	3	0.687	75.9			592 ± 23	88.1
B-6	6	0.569	62.6			561 ± 13	83.5
B-9	9	0.494	54.5			604 ± 19	89.8
B-20	20	0.514	57.3			472 ± 22	70.2
			Lamb Kidney (Na^+,K^+)-ATPase				
C-0 treated with lipase buffer	0	1.08	108		0.0786	645 ± 21	100
C-2	20	0.576	57.7		0.064	306 ± 11	47.4
C-1 (initial)	0	0.998	100		0.375	950 ± 23	100

[a] The lipid composition and enzymatic activities of the (Na^+,K^+)-ATPase samples treated as shown in Figure 1 are presented. Samples from the A series were treated with PLEP and Dns-PS/PC donor vessels and were then hydrolyzed with phospholipase C for the states times. The B series samples were run as controls and treated identically as A only without PLEP and donor vessels. The C series lamb kidney (Na^+,K^+)-ATPase was treated only with phospholipase C. The procedures for lipid and enzymatic analysis are given under Materials and Methods. [b] Average SD for PLP values ≤0.013. [c] Average SD for cholesterol content values ≤0.003.

each product.

The treatment of the (Na^+,K^+)-ATPase enzyme preparations with phospholipase C from *Clostridium welchii* was a modification of the method of Stahl (1973). *Clostridium welchii* phospholipase C, lot AD 1051A, was from Wellcome Research Labs, England. The (Na^+,K^+)-ATPase was suspended at 1.5 mg of protein/mL in 4 mM histidine, 2.5 mM $CaCl_2$, 2 mM Tris–ATP, 2 mg/mL BSA, 25% dimethyl sulfoxide (Me_2SO), and 0.1 mM $ZnCl_2$, pH 7.4, buffer. Phospholipase C was suspended in the same buffer and added to the (Na^+,K^+)-ATPase at 0.1 mg of phospholipase C/mg of (Na^+,K^+)-ATPase. The reaction was run for the times indicated in the legend of Figure 1 at 37 °C and terminated with the addition of 2 volumes of 0.1 M EGTA adjusted with Tris to pH 7.4. The samples were centrifuged at 28 000 rpm in a Spinco 40 rotor for 30 min. Samples were washed twice with 20 mM Tris-HCl and 1 mM EDTA, resuspended in SET buffer, and stored at 4 °C. Analysis of phospholipid phosphorus was performed by the method of Keenan et al. (1968). Cholesterol was measured by the technique of Bhandaru (1977), and individual phospholipid composition was determined by the two-dimensional thin-layer chromatography method of Broekhuyse (1968). Spots containing the phospholipids were located with iodine vapor, then hydrolyzed with sulfuric acid, and analyzed for phospholipid phosphorus. Protein was measured by the technique of Lowry et al. (1951) using BSA as a standard. (Na^+,K^+)-ATPase activity was measured as described by Harris & Stahl (1977) using the Lanzetta et al. (1979) procedure for determing inorganic phosphate. Phosphorylation–dephosphorylation of the (Na^+,K^+)-ATPase from [^{32}P]ATP at ice temperature was performed as described originally by Post et al. (1969) as modified by Harris & Stahl (1984). Lipids were extracted by the method of Folch et al. (1957). Neutral lipids from the lipid extract, including diacylglycerols, were separated on methanol-washed silica gel G thin-layer plates by using the two-solvent system of Freeman & West (1966). After two extractions of the scraped silica gel lanes with chloroform/methanol (2:1 v/v), quantitation was by the Amenta (1964) method. Dipalmitin was used as a standard for diacylglycerol areas on the plate.

Fluorescence measurements were made in an SLM 4800 fluorometer equipped with Glan-Thompson calcite prism polarizers and a thermostated cell compartment maintained in a nitrogen atmosphere. The samples were continuously stirred during analysis. For polarization measurements, the ratio of the fluorescence intensity of the perpendicular and parallel polarizers was sampled a minimum of 10 times at 0.25 s per sample for each reading. The temperature within the suspension was monitored with a Texas Instruments thermistor probe.

Results

Preparation and Composition of (Na^+,K^+)-ATPase. The fluorescent phospholipid Dns-PS in phosphatidylcholine (PC) donor vessels was introduced into a (Na^+,K^+)-ATPase preparation from lamb kidney by using a phospholipid exchange protein (PLEP). The phosphatidylcholine content of the (Na^+,K^+)-ATPase was increased from 37% to 56%, and 2.6% of the total phospholipid was Dns-PS. A control sample without PLEP was run in parallel (column B of Figure 1). The Dns-PS-labeled sample and the control were treated with phospholipase C for times that would remove up to 70% of the total phospholipid phosphorus but still allow the enzyme to retain activity. Evidence to support the incorporation of this lipid into the bulk-phase lipid region of the (Na^+,K^+)-ATPase will be presented below. The fluorescent properties of the probe were compared in three states: (1) incorporated within the (Na^+,K^+)-ATPase with PLEP; (2) added to the enzyme without PLEP; or (3) incorporated within the total lipid extract. This allowed an examination of the effect that the protein exerts on the bulk-phase phospholipids.

It was discovered that the buffer used during the phospholipase C hydrolysis, which contained 25% dimethyl sulfoxide (Me_2SO), removed between 80% and 85% of the cholesterol in the preparation (Table I). This was found even in zero-time samples, suggesting rapid extraction of cholesterol by the Me_2SO-containing buffer. The cholesterol content of the initial lamb kidney (Na^+,K^+)-ATPase was reduced from 0.375 mg/mg of protein to 0.078 mg/mg of protein by incubation with the buffer used for the lipase treatment (Table I). After the same treatment, the PLEP-treated enzyme

Table II: Fluorescence Properties of Dns-PS-Enriched (Na^+,K^+)-ATPase Samples[a]

sample	rel fluor intensity[b,f]	ratio of fluor intensity, (290/470)/(360/470)[c]	ratio of cor fluor intensity, (290/470)/(360/470)[d]	polarization of fluor[e] samples in SET buffer[g]	with Na^+, Mg^{2+}, and ATP[h]	% change	in 1% SDS[i]
A-0	3135	0.292	0.292	0.218	0.216	1.1	0.091
A-3	3404	0.302	0.326	0.238	0.231	2.9	0.089
A-6	3571	0.324	0.369	0.247	0.236	4.1	0.090
A-9	3632	0.325	0.377	0.245	0.238	2.9	0.089
A-20	3866	0.342	0.420	0.262	0.247	5.8	0.088
Dns-PS/PC donor vessels	2989			0.119	0.119		
donor vessels added to control enzyme B-0 without PLEP	3207			0.132	0.136		

[a] The fluorescent properties of the Dns-PS-enriched (Na^+,K^+)-ATPase samples measured at 37 °C are presented. Fluorescent measurements were made in a thermostated cell compartment maintained in a nitrogen atmosphere while the sample was continuously stirred. The protein content of the suspension was 100 μg/mL in SET buffer. Schott 470-nm long-pass filters were in the emission light paths. The following concentrations of ligands were used: 100 mM NaCl, 5 mM $MgCl_2$, and 3 mM Tris–ATP, pH 7.4. [b] Relative fluorescence intensity of Dns-PS in each (Na^+,K^+)-ATPase sample, the phospholipid donor vessels alone, and the phospholipid donor vessels added to control enzyme without PLEP (360-nm excitation and 470-nm emission wavelengths were used). [c] Ratio of potential energy transfer from the protein aromatic amino acids to Dns-PS (290/470 nm) to the Dns-PS fluorescence intensity (360/470 nm). [d] Ratio described in footnote *c* corrected for the increase in intensity shown in the second column, indicating an increase in energy transfer from sample A-0 to A-20. [e] The polarization of fluorescence of Dns-PS is shown for all the samples in SET buffer and also with NaCl, $MgCl_2$, and ATP. The difference between the above two values is given as the percent change. The polarization in 1% SDS where enzyme structure should be disrupted is also given. The data presented were from a single preparation of the individual samples and light-scattering controls measured at least in duplicate on a single occasion. [f] Average SD values ≤97. [g] Average SD values ≤0.0015. [h] Average SD values ≤0.0012. [i] Average SD values ≤0.001.

preparation contained 0.056 mg of cholesterol/mg of protein. This cholesterol reduction appeared to have little to do with the hydrolysis although an additional 3–4% of the sterol was removed when the lipase was present. The major loss of cholesterol was due to the presence of Me_2SO rather than to the treatment with PLEP. The reduction in cholesterol content imparted more distinct breaks to the temperature curves as shown below. Cholesterol is known to increase the fluidity of the less fluid phospholipid fatty acid chains and to decrease the fluidity of the more fluid fatty acid chains (Chapman, 1975).

The phospholipid contents of the Dns-PS and control (Na^+,K^+)-ATPases were reduced by treatment with phospholipase C (Table I). The Dns-PS-enriched (Na^+,K^+)-ATPase showed a greater reduction in PLP than the control sample due to the greater PC content of the former. The Dns-PS content was not changed by phospholipase C as determined by radioactivity of the products (data not shown) and by fluorescence intensity measurements. Phospholipase C treatment increased the proportion of Dns-PS in the total phospholipids from 2.6% to 8.2%. The products of phospholipase C hydrolysis are diacylglycerols, or sphingosine, and the phosphorylated head group, choline or ethanolamine. The latter are water soluble and were removed by the washing procedures. The buffer that removed cholesterol from the membrane also removed 35% ± 13% of the diacylglycerol produced by the enzymatic hydrolysis. Assuming a molecular weight of 351 for the diacylglycerol, the initial (Na^+,K^+)-ATPase preparation contained 0.22 μmol of diacylglycerol/mg of protein. The control sample (C-0, Table I) exposed to Me_2SO contained 0.14 ± 0.02 μmol of diacylglycerol/mg of protein. The (Na^+,K^+)-ATPase treated with phospholipase C in the Me_2SO buffer contained 0.60 ± 0.08 μg of diacylglycerol/mg of protein. The theoretical amount of the diacylglycerol was calculated from the endogenous amount, and the conversion of phospholipid to diacylglycerol was 0.92 μmol/mg of protein. Greater than one-third of the neutral lipid formed by phospholipase C treatment was removed by the suspending buffer.

The (Na^+,K^+)-ATPase specific activity was minimally affected by the altered phospholipid content as the control samples (series B) and Dns-PS ATPase samples (series A) had similar activities though both were reduced by the experimental manipulations (Table I). As reported by DePont et al. (1978), it was found that up to 40% of the phospholipid could be hydrolyzed with only a 10% reduction in enzyme activity. The hydrolysis of 57% of PLP caused 30% loss of activity while hydrolysis of 68% of the phospholipids produced a 55% loss of enzyme activity. Thus, a series of enzymatically active (Na^+,K^+)-ATPase samples with a fixed fluorescent probe content, but with reduced cholesterol and reduced phospholipid contents, were available (Table I).

Fluorescence Excitation Spectra. A major fluorescent excitation peak at 356 nm characteristic of the Dns group was found in the excitation spectra of all the Dns-PS preparations (Figure 2). A second excitation peak at 288 nm was present when the fluorescent phospholipid was incorporated into the (Na^+,K^+)-ATPase by using the phospholipid exchange protein. This peak was absent in the donor vessels alone and also was absent when the Dns-PS/PC donor vessles were added to the control (Na^+,K^+)-ATPase at concentrations equivalent to the amount of Dns-PS incorporated. A decrease in protein intrinsic fluorescence, measured by exciting at 290 nm with an emission of 340 nm, was observed when the (Na^+,K^+)-ATPase had Dns-PS incorporated via PLEP. This would indicate that energy transfer in the excited electronic state occurs between the aromatic amino acids of the protein and the Dns group in the lipid region.

Fluorescence Intensity. The fluorescence intensity of the Dns group has been reported to be very sensitive to the polarity of its immediate environment (Waggoner & Styer, 1970). The relative fluorescent intensity of the Dns group on phosphatidylserine increased as phospholipid was removed from the enzyme preparation (Table II). A maximum increase in intensity of 23% was observed when 68% of the phospholipid was removed (column 2, Table II). All samples contain the same amount of Dns-PS as determined by measurement of the radioactivity of Dns-PS in each sample and also by disruption of the membrane structure with 1% SDS. In the latter case, the fluorescence intensities of all five samples were identical within experimental error. The fluorescent value in 1% SDS was 45% of the intensity when the probe was present in the enzyme preparation. The increase in fluorescence intensity indicated that the probe was in an increasingly nonpolar environment as the phospholipid content of the preparation was reduced.

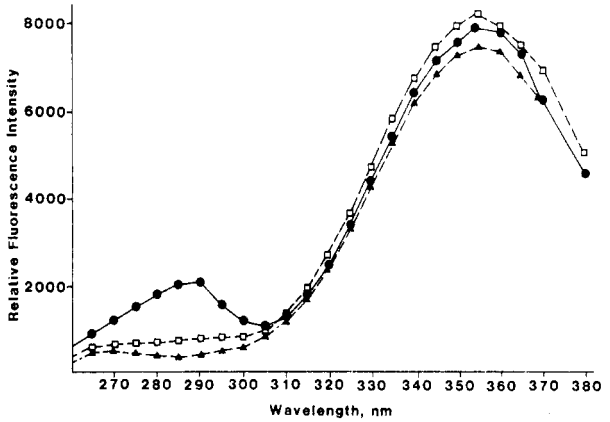

FIGURE 2: Fluorescence excitation spectra of Dns-PS incorporated into (Na^+,K^+)-ATPase with PLEP (●) or mixed with control (Na^+,K^+)-ATPase (□) or donor 90% PC/10% Dns-PS vessels (▲). The protein concentration was 100 μg/mL SET buffer, and the lipid concentration was matched to the incorporated Dns-PS level from the data in Table I. Emission was monitored through a Schott K.V. 470-nm long-pass filter.

A method to determine if the Dns-PS was in close proximity to the (Na^+,K^+)-ATPase when the phospholipid content was reduced was needed. Comparison of the fluorescence intensity due to energy transfer from protein to probe, exciting at 290 nm with emission at 470 nm, and the fluorescence intensity at the probe's excitation maximum, 360 nm, was used to examine this possibility.

Table II, column 3 presents the relation of the relative peak heights of the 290/470-nm peak divided by the 360/470-nm peak as the phospholipid content was reduced. In the initial Dns-PS-enriched (Na^+,K^+)-ATPase, the 290-nm peak was 29% of the height of the major excitation peak. With the progressive hydrolysis of phospholipids, this ratio increased in a stepwise fashion to 34% of the 360/470-nm peak height. The fluorescence intensity measured by exciting at 360 nm with emission at 470 nm also increased with removal of phospholipids. When corrections are made for this increase, the ratio of the peak heights increases from 29% to a final value of 42%. This indicates that the fluorescent portion of the lipid came closer to the aromatic amino acids within the protein portion of the preparation as the phospholipid content was reduced.

Polarization of Fluorescence. The polarization of fluorescence provides information about the probe's ability to rotate within the plane of the membrane. This parameter is independent of fluorescent intensity and decreases within a more fluid environment and increases within a less fluid one. This fluorescent parameter permits a clear distinction between the various environments in which the probe may be located. When Dns-PS was present in the donor lipid vessels alone, its polarization of fluorescence was 0.119 (Table II, column 5). When control enzyme and donor phospholipid vessels were simply mixed together at the same concentration as the Dns-PS incorporated into the enzyme with PLEP, the polarization of fluorescence of the Dns group was 0.132. The increase in polarization of fluorescence could indicate that a portion of the phospholipid vessels was adhering to the surface of the membrane preparation and had reduced freedom of rotation. The Dns-PS enriched (Na^+,K^+)-ATP prepared with PLEP had a polarization of fluorescence of 0.218 at 37 °C.

An additional increase in polarization of fluorescence was observed upon hydrolysis of endogenous phospholipids by phospholipase C. The polarization of Dns fluorescence increased to a maximum value of 0.262 when 68% of the phospholipid was hydrolyzed (sample A-20). The values (given in column 5 of Table II) were all measured at 37 °C in 50 mM Tris-HCl, 1 mM EDTA, and 250 mM sucrose, pH 7.4. To determine if a change in conformation of the enzyme induced by ligands would have any affect on the polarization of the probe, 100 mM NaCl, 5 mM $MgCl_2$, and 3 mM Tris–ATP were added to the suspension. Resultant values are reported in column 6 of Table II. A decrease in polarization of the Dns groups was observed in all cases. However, a majority of this decrease was due to an ionic strength effect seen with the addition of 100 mM monovalent cation. Potassium could be substituted for sodium with an identical effect on polarization. A possible interpretation is that this effect could be due to binding of cations to charged species within the membrane structure, causing a relaxing of charge repulsion within the sample.

Although the changes observed between columns 5 and 6 are only 1–6% of the initial polarization value (Table II, column 7), there was a greater change in polarization after ligand addition when the bulk-phase phospholipid content was reduced. With reduction of the phospholipids, the enzyme should be closer to the probe and should have a greater measurable effect on the polarization of fluorescence of Dns-PS.

To examine the proposal that the changes in polarization of Dns-PS fluorescence after hydrolysis of endogenous phospholipids were due to organization of the lipid bilayer of the preparation, all samples were disrupted with 1% SDS. It was found that the polarization of fluorescence of all samples dropped to the same value, 0.0899 ± 0.0002 (column 8, Table II).

Temperature Profile of Polarization of Fluorescence and (Na^+,K^+)-ATPase Activity. The temperature of the phase transition from fluid to gel states of the phospholipid fatty acids has been considered as a measure of the fluidity of the hydrocarbon core of the bulk-phase lipids. The temperature profiles of the polarization of fluorescence of the Dns-PS-enriched (Na^+,K^+)-ATPase before and after phospholipase C treatment are shown in Figure 3A. The temperature profiles of the total lipids extracted from those preparations are shown in Figure 3B. The analysis of the data underlying this figure is presented in Table III. The temperature of the transition, T_m, from gel to fluid states of the untreated Dns-PS-enriched (Na^+,K^+)-ATPase was 32 °C. When 30–40% of the phospholipid was hydrolyzed, T_m decreased to 28 °C. With 57% hydrolysis of phospholipid, the T_m was 23 °C, and with the maximum 68% decrease in phospholipid (sample A-20), the T_m was 20 °C. In contrast, the total lipid extract from each of these samples at a concentration identical with that of the phospholipid within the enzyme showed no decrease in the transition temperature with lipase treatment. All five samples were within the same range as the untreated sample, 30 ± 2 °C. This would indicate that the protein within the membrane was exerting a fluidizing effect on the bulk-phase phospholipids. One possible explanation for the difference between the behaviors of intact membranes and the lipid extract would be that the phospholipids with higher transition temperatures, i.e., those with longer fatty acid chains or saturated fatty acids, were preferentially associating with the protein in the membrane. This would leave the phospholipids with shorter chains or more unsaturated chains in the bulk phase, which would then have a lower transition temperature.

(Na^+,K^+)-ATPase activity was measured at different temperatures to establish if the altered phospholipid composition had an effect on the T_m of the enzymatic reaction as well as

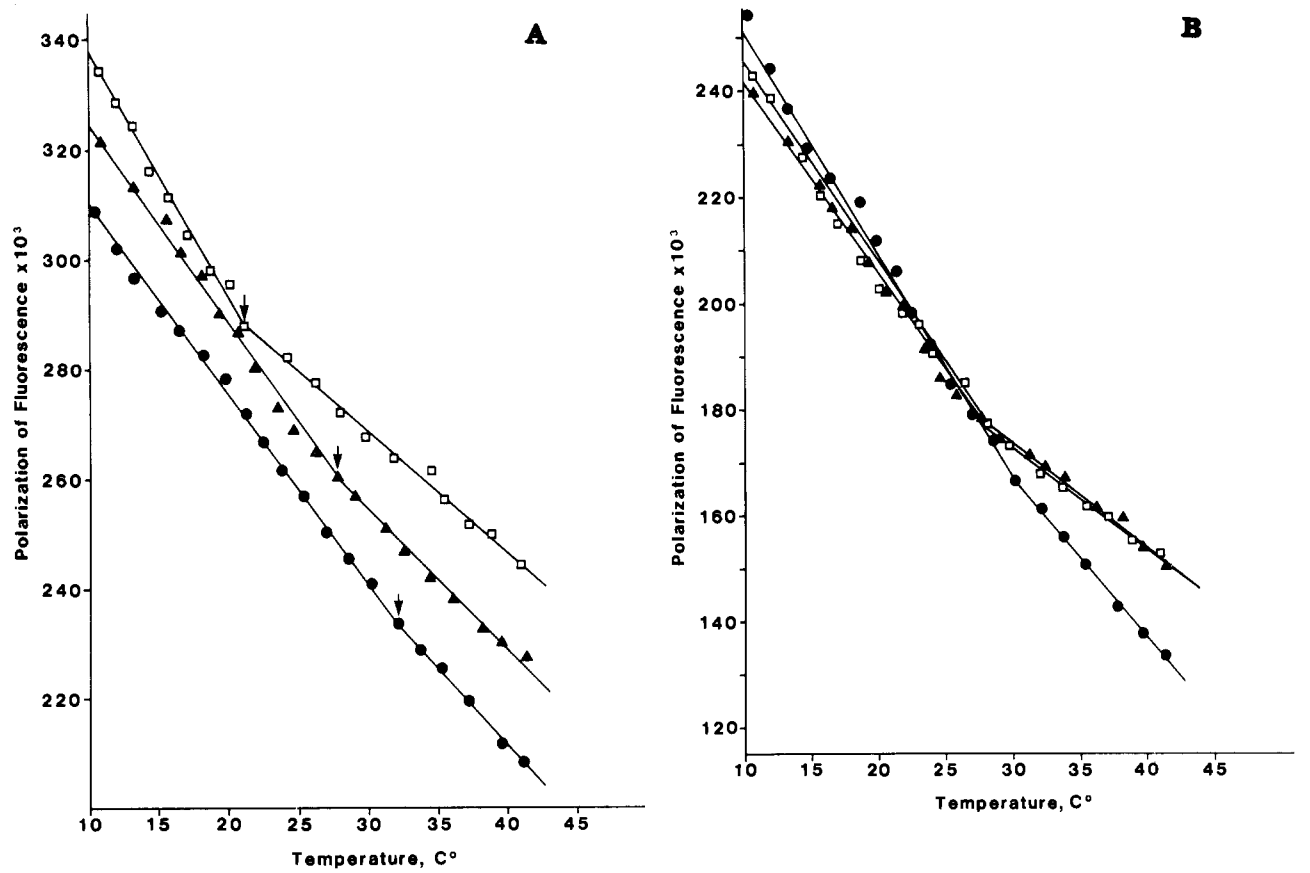

FIGURE 3: Polarization of fluorescence of Dns-PS-enriched (Na^+,K^+)-ATPase samples (A) and total lipid extracts of the (Na^+,K^+)-ATPase (B) measured at increasing temperatures. All samples were prepared at 100 μg of protein/mL of SET buffer or the equivalent lipid concentration as in Table I. Samples were cooled to approximately 10 °C and were then heated at approximately 0.5 °C/min to approximately 40 °C. Temperature was measured with a Texas Instruments thermistor within the cuvette. Polarization of fluorescence measurements was made by taking a minimum of four separate measurements of 10 × 0.25 s per measurement. The excitation wavelength was 360 nm with a Schott 470-nm long-pass filter in the emission light paths. Initial corrections were made for light scatter by using the B series of control enzyme (Table I). A-0 (●); A-6 (▲); A-20 (□).

Table III[a]

(A) Transition Temperatures for Polarization of Fluorescence

sample	T_m (°C) enzyme	T_m (°C) lipid extract
A-0	32.8	30.4
A-3	27	29.5
A-6	28	29
A-9	23.4	28.5
A-20	20.0	30.2

(B) Arrhenius Activation Energies of (Na^+,K^+)-ATPase

T_m (°C)	E_a (kcal/mol) below T_m	E_a (kcal/mol) above T_m
32.6	21.2	16.5
29	19.5	14.1
28.9	22.2	14.0
28.7	22.4	13.8
24.8	19.8	13.3
	av 21.0 ± 1.3	14.3 ± 1.2

[a] The transition temperatures, T_m, were determined from the plot of the polarization of fluorescence vs. temperature shown in Figure 3A,B. The T_m values for the enzymatic activity were derived from a plot of the natural logarithm of the specific activity vs. $1/T$ (with temperature in degrees kelvin). The Arrhenius activation energies were derived from the slopes of the stated lines by using the relation $-E_a = R\mathrm{d}(\ln v)/\mathrm{d}(1/T)$.

to assess the likelihood that preferential association of long-chained phospholipids with the enzyme occurred. The tabulation of data for all samples is presented in Table IIIB. These data illustrate that the T_m for the enzyme reaction decreases with the hydrolysis of phosphatidylcholine and sphingomyelin in a manner similar to the decrease in the polarization of fluorescence of Dns-PS within the lipid region of the preparation. The possibility of longer chain phospholipids associating with the enzyme is discounted, and it is seen that the lipid influences the enzymatic properties of the (Na^+,K^+)-ATPase and the enzyme affects the fluidity of the lipid region.

The Arrhenius activation energies, E_a, of the Dns-PS-enriched (Na^+,K^+)-ATPase samples are shown in Table III. There were very small differences in the activation energies above or below the T_m of the samples. Below the T_m, all the samples had almost the same activation energy of 21 ± 1.3 kcal/mol. Above the T_m, the values were also very similar, 14.3 ± 1.2 kcal/mol. These data would suggest that either above or below the T_m the same fluidity exists within the bulk-phase phospholipids under any of the conditions examined. The major effect of reduction of the phospholipids is a lowering of the T_m, not alteration of the Arrhenius activation energies.

Fluorescent Lifetimes. The analysis of fluorescent lifetimes of the Dns probe within the enzyme preparation provides additional insight into the environment of the probe. The procedure used in this study measures the phase shift and modulation of the exciting signal at three modulation frequencies. Both parameters yield measured lifetimes that are affected by the modulation frequency. By means of the Weber algorithm, it is possible to resolve these measured lifetimes into their original components. This algorithm can be used to resolve either a homogeneous population of fluorophores or

Table IV: Fluorescence Lifetimes for Dns-PS in Membranes[a]

sample	measured lifetimes (ns) at 6 MHz	8 MHz	30 MHz		heterogeneity analysis 6 vs. 18 MHz	6 vs. 30 MHz	18 vs. 30 MHz
A-0							
phase	12.47 ± 1.01	10.58 ± 0.51	9.66 ± 0.18	τ_1	5.15	5.47	5.84
modulation	14.15 ± 0.86	12.89 ± 0.21	12.02 ± 0.14	τ_2	15.15	15.08	14.93
				α_2	0.775	0.769	0.728
A-20							
phase	12.50 ± 0.26	11.03 ± 0.11	9.72 ± 0.43	τ_1	5.67	5.19	4.83
modulation	14.34 ± 0.30	12.96 ± 0.06	12.16 ± 10.07	τ_2	14.68	14.81	15.31
				α_2	0.782	0.793	0.833
donor vessels (PC/Dns-PS)							
phase	11.84 ± 0.17	11.35 ± 0.06	11.06 ± 0.06	τ_1	−3.92	4.46	−5.65
modulation	12.51 ± 0.46	12.65 ± 0.06	11.86 ± 0.15	τ_2	12.32	12.36	12.07
				α_2	0.974	0.947	0.975

[a] The fluorescence lifetimes of Dns-PS in membranes were measured at 37 °C in SET buffer, at 100 μg of protein/mL or equivalent phospholipid concentration, under N_2 with continuous stirring. Excitation was at 360 nm with a Schott K.V. 470-nm long-pass filter in the emission light path. Oyster glycogen was used as a light-scattering reference. The heterogeneity analysis using the Weber algorithm was done by using a program supplied by the SLM Instrument Co. τ_1 is the lifetime (in nanoseconds) of the short-lifetime component, τ_2 the lifetime (in nanoseconds) of the long-lifetime component, and α_2 the proportion of the fluorescent intensity τ_2 lifetime.

a two-component system. More than two components cannot be resolved. In these cases, it can only be concluded that a "heterogeneous" population exists.

Analysis of the fluorescent lifetimes of the excited state of the Dns group after incorporation into the (Na^+,K^+)-ATPase preparation suggests that two populations with different lifetimes exist (Table IV). Most of the fluorescence intensity, 75.7 ± 2.5%, in the A-0 sample had a fluorescent lifetime of 15.05 ± 0.11 ns while the remaining 25% of the intensity had a shorter lifetime of 5.48 ± 0.11 ns. The shorter lifetime could be due to static quenching by groups adjacent to the probe. This heterogeneity did not change with hydrolysis of phospholipid in the preparation. For example, for A-20 in which the phospholipid content was reduced by 68%, 80.2% of the fluorescence intensity had a lifetime of 14.93 ± 0.42 ns, and the remaining 20% had a shorter lifetime of 5.23 ± 0.35 ns. Other samples had similar values. In contrast, the total lipid extract of these samples could not be resolved into two components by the Weber algorithm. These results again indicate that the protein exerts a major ordering influence on the structure of the lipid bilayer.

Fluorescence Quenching by Iodide. The dynamic quenching of fluorescence by iodide can indicate the accessibility of the fluorophore to that anion as well as the rate at which the interaction occurs. The theory derived by Lehrer (1971), though used for multiple fluorescing groups within proteins, may be applied to other multicomponent systems. The quantity "f_a" is the proportion of the total fluorescent population accessible to the quencher. As shown in Figure 4 and Table V, this quantity remains essentially constant for the five Dns-PS-enriched (Na^+,K^+)-ATPase samples with or without phospholipase C treatment. About 44% of the total Dns-PS population can be quenched by I^-. The rate at which this collisional quenching occurs changes as the phospholipid content decreases. A marked decrease in the second-order rate constant, k_3, occurs with the removal of PC and SM. For the initial untreated sample, the rate constant for the quenching at 37 °C was 2.62×10^8 s^{-1} M^{-1}. After hydrolysis of 68% of the phospholipids, that rate had decreased to 1.43×10^8 s^{-1} M^{-1}. The Dns groups on the PS have been shown to be located within the glycerol region of the phospholipid (Harris & Stahl, 1976). While the same proportion of the probe population was available to I^-, the decreased rate constant reflects structural alteration in the lipid domain of the membrane such that the fluorescent groups either are in a more nonpolar environment or are shielded from iodide by other anionic changes after phospholipase C treatment. The former interpretation is favored; it is consistent with the observed increase in fluorescent intensity as phospholipid content is decreased.

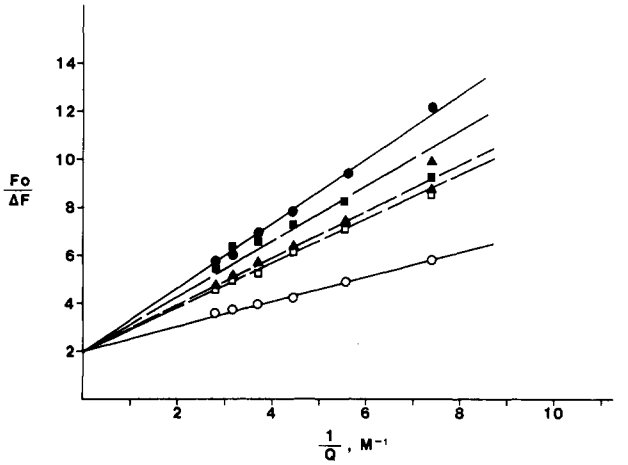

FIGURE 4: Quenching of fluorescence of Dns-PS within (Na^+,K^+)-ATPase. A sample of 100 μg of protein/mL of SET buffer with 100 mM NaCl was quenched by addition of concentrated NaI with results corrected for any ionic strength effects produced by addition of an equivalent concentration of NaCl to a matched sample. The sample was excited at 360 nm with a Schott 470-nm long-pass filter used in the emission light paths. A-0 (○); A-3 (□); A-6 (▲); A-9 (■); A-20 (●).

Table V: Fluorescence Quenching by Iodide Ion[a]

sample	f_a	K_Q	τ_0 (ns)	k_3 (s^{-1} M^{-1})
A-0	0.442	3.28	12.5	2.62×10^8
A-3	0.442	2.53	12.5	2.02×10^8
A-6	0.441	2.22	12.5	1.78×10^8
A-9	0.442	2.02	12.5	1.62×10^8
A-20	0.444	1.79	12.5	1.43×10^8

[a] The fluorescence quenching by iodide ion of Dns-PS (Na^+,K^+)-ATPase samples is presented. The quantity f_a is that fraction of the total population of fluorescing species that is available to the quencher, K_q is the quench constant, τ_0 is the fluorescent lifetime, and the second-order rate constant, k_3, is derived from the Lehrer modification of the Stern–Volmer relationship: $F_0/\Delta F = 1/[Q]f_a\tau_0k_3 + 1/f_a$. The value of 12.5 ns for τ_0 was derived from the data in Table IV by weighing the fluorescence intensities and two lifetimes. The distribution of intensities and lifetimes did not change with phospholipase C treatment.

Dephosphorylation of (Na^+,K^+)-ATPase. The increase in the polarization of fluorescence of the Dns group in the (Na^+,K^+)-ATPase samples after phospholipase C treatment

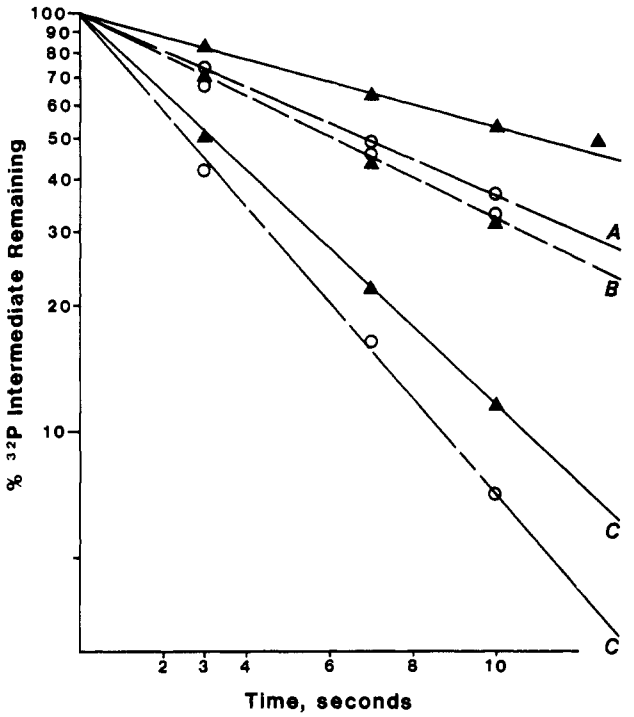

FIGURE 5: Rate of dephosphorylation of the ^{32}P-phosphoprotein intermediate of control (Na^+,K^+)-ATPase and phospholipase C treated (Na^+,K^+)-ATPase. At zero time, CDTA was added to halt phosphorylation by chelation of Mg^{2+}. Cl_3CCOOH was added to stop the dephosphorylation at the times indicated. Control (Na^+,K^+)-ATPase, sample C-0 of Figure 1, is represented by (○), while the phospholipase C treated enzyme, sample C-20 (Figure 1), is shown by (▲). The A series had CDTA only added at zero time, the B series CDTA + ADP, and the C series CDTA + K^+. Details of the procedure are given under Materials and Methods.

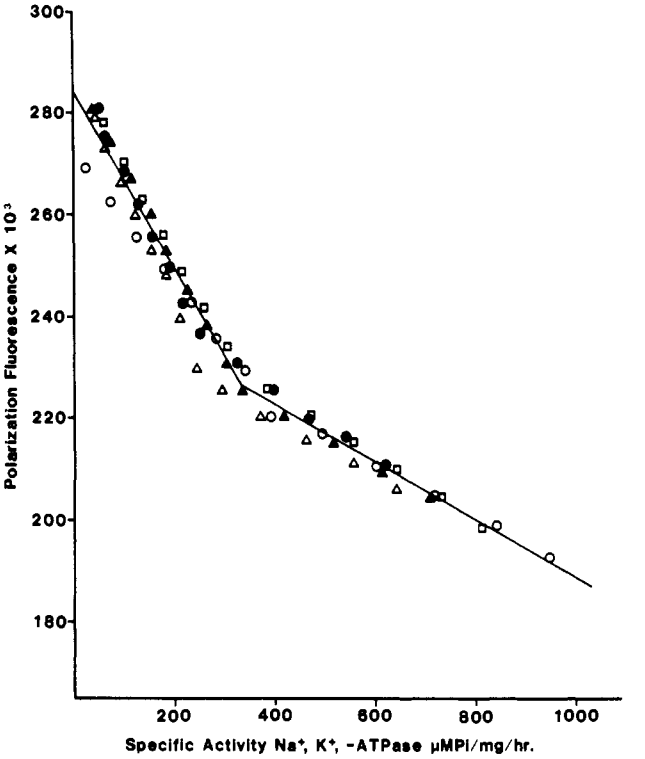

FIGURE 6: Correlation between specific activity of (Na^+,K^+)-ATPase and polarization of fluorescence of Dns-PS within enzyme samples between 14 and 43 °C. The polarization of fluorescence for the five Dns-PS-enriched (Na^+,K^+)-ATPase samples was taken from the data shown in Figure 3A. The enzyme activity data for these samples were from Table III. This figure demonstrated that the same enzyme activity is found at the same polarization value (or bilayer fluidity) for any of the samples irrespective of their phospholipid content. A-0 (○); A-3 (●); A-6 (△); A-9 (▲); A-20 (□).

and the decrease in enzyme activity could have been the expression of the same physical phenomenon such as a reduced fluidity of the phospholipid of the preparation. A more rigid lipid region could restrict the enzyme conformational changes necessary for activity. To examine this possibility, the steady-state levels of the phosphorylated intermediate of the (Na^+,K^+)-ATPase and the rate of dephosphorylation of the [^{32}P]phosphoprotein intermediate were examined (Harris & Stahl, 1984). Because of the amount of material required, the "C" series of samples (Figure 1), the native enzyme treated with phospholipase C, rather than the "A" series of samples (containing Dns) were used.

The control (Me_2SO treated) and the phospholipase C treated enzyme preparations bound slightly more ^{32}P than the initial enzyme preparation, the control (2100 pmol/mg of protein), the phospholipase C treated preparation (2000 pmol/mg), and the initial enzyme (1500 pmol/mg). The most significant compositional difference between the initial and control samples was the loss of 85% of the cholesterol in the control enzyme sample. The initial binding of ATP and phosphorylation of the enzyme were not inhibited but rather stimulated by the treatment with Me_2SO, and no significant difference of steady-state ^{32}P intermediate levels was observed between the control and phospholipase C treated sample. The rate of dephosphorylation of the preparations differed significantly between the samples (Figure 5). The basal rate of dephosphorylation in the presence of CDTA (Harris & Stahl, 1984) for the phospholipase C treated sample was significantly lower than that for the control. The phosphorylated intermediate of the control did not use ADP as a phosphate acceptor in the reverse reaction of the (Na^+,K^+)-ATPase; i.e., the rates of dephosphorylation in the presence or absence of ADP were identical. In contrast, the phosphorylated intermediate of the phospholipase C treated sample did react with ADP. Both the control and phospholipase C treated samples were potassium sensitive. In the presence of CDTA, 25 mM KCl caused a rapid loss of the phosphorylated intermediate. However, the rate of loss of the ^{32}P intermediate was lower in the phospholipase C treated sample than in the control.

These data indicate that the initial steps in the (Na^+,K^+)-ATPase reaction, the binding of the substrate and phosphorylation of the enzyme, were not adversely affected by the lipase treatment. The latter steps of the reaction that require conformational changes in the enzyme had decreased rates of dephosphorylation after removal of phosphatidylcholine and sphingomyelin.

Relation between (Na^+,K^+)-ATPase Activity and the Polarization of Fluorescence of Dns-PS. If the fluidity of the phospholipid bilayer was the limiting factor in the enzyme reaction, then one would expect the enzyme activity to be the same at identical values of polarization of fluorescence of the Dns group in the bilayer for each of the samples. To explore this possibility, data from temperature vs. polarization of fluorescence and temperature vs. enzyme specific activity studies were obtained (Figure 6). Excellent correlation for the five Dns-PS-enriched (Na^+,K^+)-ATPase samples was found between enzyme activity and polarization of fluorescence of the Dns-pS within the preparations. With enzyme specific activity between 25 and 325 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, the correlation coefficient was 0.956 for $n = 40$, while at higher enzyme activities, the correlation was 0.973 for $n = 25$. (Na^+,K^+)-ATPase specific activities of over 600 μmol of P_i

(mg of protein)$^{-1}$ h $^{-1}$ were obtained at temperatures above the normal 37 °C assay temperature.

This indicates that the decreases in enzymatic activity shown in Table I for samples with reduced phospholipid content were the result of decreased fluidity of the lipid matrix. Any of the five samples at a specific polarization value had identical (Na^+,K^+)-ATPase activity. This also indicates that the enzyme activity was independent of the total phospholipid content. These data support the concept that the phospholipid bilayer affects the activity of the (Na^+,K^+)-ATPase in a nonspecific manner by providing a matrix of adequate fluidity for the enzyme to function.

There is likely a minimum amount of phospholipid that is required to have the enzyme retain its active conformations, but the phospholipid content retained in this study is above that minimum value.

Discussion

The nonspecific phospholipid exchange protein appears to be useful for introducing fluorescent phospholipids, e.g., Dns-phosphatidylserine, into the purified (Na^+,K^+)-ATPase. Mild conditions were used, and (Na^+,K^+)-ATPase enzyme activity was retained. Previous studies suggest that the probe is incorporated into the lipid bilayer of the preparation (Muczynski et al., 1983; Muczynski & Stahl, 1983). The proportion of the probe in the phospholipid bilayer was kept low (i.e., 2–8% of the total phospholipids) to minimize the possibility of the fluorescent moiety perturbing the lipid structure and to minimize nonspecific adhesion of donor vessels containing Dns-PS with the (Na^+,K^+)-ATPase preparation. Several parameters, including fluorescence intensity, excitation spectra (which showed energy transfer from the aromatic amino acids of the protein to the Dns group), and polarization of fluorescence, measured for the Dns-PS probe incorporated into the enzyme preparation, differed distinctly from those measured for the probe either in the donor PC/Dns-PS vessels or in the total lipid extract. These parameters also differed from those of a simple mixture of lipid donor vessels and with the enzyme preparation without the exchange protein.

A consideration of protein tertiary structure suggests that the nonpolar amino acid residues, including the fluorescent aromatic amino acids, would likely be located in a nonpolar environment at the intramembrane phospholipid bilayer. It has been shown previously (Harris & Stahl, 1976) that the Dns group in Dns-phosphatidylserine resides in the glycerol region of the phospholipid structure below the head groups and above the fatty acid region; this implies the probability of interaction between the fluorescent Dns groups with those aromatic amino acids.

Phospholipase C treatment of the Dns-PN-enriched (Na^+,K^+)-ATPase preparation caused modification of the phospholipid bilayer structure as well as decreased enzyme activity. The fluorescent intensity, excitation spectra, and polarization of fluorescence all pointed to greater association of the phospholipid probe with the protein as the phospholipid content was reduced. In contrast, the distribution of fluorescence lifetimes of the Dns-PS probe was not changed by the reduced phospholipid content.

This result generally agrees with the information obtained by the quenching of the Dns fluorescence by I^-. The same proportion of the total Dns-PS population, 44%, could be quenched by I^- in all the samples, but the second-order rate constant was reduced as the phospholipid content was reduced. Since phosphatidylcholine and sphingomyelin were hydrolyzed by phospholipase C and both have neutral head groups, the proportion of phospholipids with charged head groups (PS and PE) in the preparation was increased. This would have a direct effect on the rate at which I^- could approach the Dns-PS in the phospholipid structure. The 1.5–2 mol of Dns-PS/mol of enzyme that was statically quenched in situ determined by lifetime analysis was also likely in the group that could not be quenched by iodide.

It is possible to approximate the molecular dimensions between the components of the (Na^+,K^+)-ATPase by using the available quantitative data on protein, phospholipid, and cholesterol contents and the molecular dimensions of these individual components. The phospholipid bilayer is between 40 and 50 Å in thickness. The area of the polar head groups of a "typical phospholipid" is between 40 and 60 Å^2, which would imply a diameter of 8 Å (Chapman, 1975). Several reports (DeGuchi et al., 1977; Cantley et al., 1983) indicate that the (Na^+,K^+)-ATPase transmembrane height is between 70 and 90 Å. A substantial portion of the protein molecule extends beyond the lipid bilayer (Nicholas, 1984). In the present case, it makes no difference if the extra bilayer regions are uniformly or asymmetrically distributed between the inner and outer membrane faces. The diameter of 75 Å for a 280 000-dalton structure derives from the electron microscopic study of DeGuchi et al. (1977). Assuming a cylindrical structure of the protein through the lipid bilayer, the protein would have a minimum circumference of 236 Å. If one assumes that phospholipidhead groups pack in a cylindrical annulus surrounding the protein, one can calculate the number of phospholipids in each concentric ring. The first ring would contain 36 phospholipids per side of the bilayer for a total of 72,[2] the second ring, 84, the third, 97, and the fourth, 108. The compositional data (Table I) showed that 322 mol of phospholipids was present per mole of enzyme, which would complete four concentric rings around the enzyme. This would mean that if the probe phospholipid were in the fourth layer of the preparation, it would be a minimum of 32 Å from the outer surface of the protein. With these approximate dimensions and proportions, the center to center distance between proteins would be 139 Å while the thickness of the phospholipid bilayer from protein edge to edge would be 64 Å. The phospholipid bilayer would comprise 70.9% of the area of the plane of the membrane. It would be less than the actual area due to the probable oblate ellipsoidal shape of the enzyme extending beyond the bilayer plane (Nicholas, 1984).

After hydrolysis of the phospholipid of phospholipace C and partial removal of the neutral lipid products, the center to center distance between the proteins should have been substantially reduced. Since Dns-PS was not hydrolyzed, the probability of having the probe in close proximity to the protein was increased.

The increase in polarization of the Dns group that occurred upon removal of phospholipid could be due either to more efficient packing of the head groups of the phospholipids remaining in the structure or to reorganization of the fatty acid tails. Since the major reduction in cholesterol, which should be located within the fatty acid region of the bilayer, had no effect on the polarization of fluorescence of the Dns group in the glycerol region, it would appear that the probe is "observing" a change in the packing of the head groups.

Several proposed models for the location of cholesterol within the bilayer show it interdigitated between the fatty acids of the phospholipids and occupying the space near the center of the bilayer where the fatty acids from either side of the

[2] Brotherus et al. (1981) calculated between 60 and 65 phospholipid contact sites per $\alpha_2\beta_2$ unit of the (Na^+,K^+)-ATPase.

bilayer terminate. The cholesterol depletion of the lipid region by treatment with Me_2SO must cause a decrease in the internal volume of the bilayer. However, cholesterol must have little or no effect on the structural organization of the glycerol region of the phospholipid where the fluorescent probe was located as no change in the fluorescence parameters of the Dns probe was observed before and after the Me_2SO treatment. This is also indicative of the region that the Dns probe is monitoring. Cholesterol has been shown to modify the structural characteristics of the fatty acid regions by making the gellike regions more fluid and the fluid regions more gellike (Chapman, 1975), so cholesterol depletion should cause more distinct temperature transitions between these two phases of the bilayer structure.

A 35–40% loss of (Na^+,K^+)-ATPase activity was found after the cholesterol loss and washing procedures. This result is similar to the report of Peters et al. (1981a) in which the (Na^+,K^+)-ATPase preparation was extracted with hexane to remove all the steroid components. After this treatment, the preparation retained 60% of the original activity. These authors also used a cholesterol oxidase to oxidize all the cholesterol in the preparation which caused a 15% loss of enzyme activity. They concluded that cholesterol was not essential for (Na^+,K^+)-ATPase activity, which is supported by the present study.

The transition temperature from gel to fluid state of the phospholipid bilayer determined by the polarization of fluorescence of the Dns-PS groups was essentially the same value (30 ± 2 °C) for the phospholipid extracts for all five samples. This indicates that the acyl groups in the phospholipids removed by the lipase treatment had a similar distribution of chain lengths and unsaturation as those remaining in the bilayer after treatment. When the probe was incorporated into the lipid bilayer of the enzyme preparation, the protein had a pronounced effect on the transition temperature. The initial "A-0" sample had a slightly higher transition temperature than the total lipid (32.8 °C), but with the progressive removal of bulk-phase phospholipids, the T_m decreased until with 68% removal of phospholipids it was 20 °C. Initially, this result appeared to conflict with the polarization data showing a decrease in fluidity with the hydrolysis of phospholipid. The probe is located within the glycerol region of the phospholipid which has more organized packing of polar head groups of the phospholipids, but this appears to allow the fatty acid acyl groups greater heterogeneity which was amplified by the reduction in cholesterol. Thus, the protein affects the packing organization of the polar groups which may, in part, be due to the protein groups which, in turn, creates a less organized hydrocarbon region.

The enzyme activity determined as a function of temperature (Table IIIB) followed the same decrease in transition temperature that was observed for the polarization of fluorescence of the Dns group. The T_m for the A-0 sample was 32 °C for the enzyme reaction; the next three samples showed a drop in transition temperature to 29 °C, while in the sample with the most phospholipid removed, the transition temperature was 24 °C. It would seem reasonable that the same phenomenon, greater heterogeneity of the acyl region of the bilayer, caused the decrease in T_m of both enzyme activity and polarization of fluorescence.

To examine the influence that the phospholipid matrix exerted on the activity of the (Na^+,K^+)-ATPase, a means of evaluating any change in fluidity of the lipid bilayer was required. The fluorescent lipid probe, Dns-PS, was introduced into the enzyme preparation under mild conditions using a nonspecific phospholipid exchange protein. Fluorescence polarization was followed in a series of samples with different lipid bilayers which were produced by limited enzymatic hydrolysis of endogenous phospholipids of the enzyme preparation by a lipase. This treatment allowed the enzyme and fluorescent probe to remain unaltered in each sample while the bulk phospholipid content was reduced. The temperature was varied to change the polarization of each sample in order to modify the fluidity of the phospholipid matrix and the (Na^+,K^+)-ATPase specific activity. With this information, Figure 6 was constructed. The data indicate that the activity of the (Na^+,K^+)-ATPase is dependent upon the polarization of fluorescence fluidity of the lipid bilayer in a nonspecific manner. At a given polarization value of the Dns-PS probe, all the samples had the same enzyme activity. Each was measured at a different temperature but resulted in the same state of fluidity for the enzyme to function. For example, the sample with all endogenous phospholipids, A-0, had a specific activity of 543 μmol (mg of protein)$^{-1}$ h^{-1}, and the polarization of Dns-PS was 0.218 at 37 °C. The sample with 68% reduction in phospholipid content, A-5, had a specific activity of 255 μmol (mg of protein)$^{-1}$ h^{-1} at 37 °C and a polarization of 0.262. For sample A-0 to have a polarization of 0.262, it was necessary to reduce the temperature to 23 °C. At that temperature, the enzyme activity of sample A-0 was 249 μmol (mg of protein)$^{-1}$ h^{-1} which was essentially the same as that for sample A-5 at 37 °C. This correlation between enzyme activity and polarization at the probe in the phospholipid bilayer was found with all samples. One interpretation of these results is that the resistance to conformational changes required for the (Na^+,K^+)-ATPase to function could be modulated by the surrounding lipid environment. This is confirmed by the data presented in Figure 6.

The data indicated that the decrease in the enzyme activity with reduction in phospholipid content of the (Na^+,K^+)-ATPase preparation may be due to the decrease in fluidity of the phospholipid region (Figure 5). The latter was determined by the increase in polarization of Dns-PS as the phosphatidylcholine and sphingomyelin content was reduced by phospholipase C in the samples. The level of formation of the phosphorylated intermediate was not affected by the phospholipid reduction. This was anticipated as this step in the enzyme reaction would depend upon the ability of the substrate, ATP, to reach and bind to the active site of the enzyme. The later steps of the (Na^+,K^+)-ATPase reaction scheme that led to the dephosphorylation of the protein were slower for phospholipase C treated samples than for control samples. This decrease in dephosphorylation rate could be due to increased viscosity of the phospholipids surrounding the enzyme, retarding the conformational changes necessary for dephosphorylation to occur. The reaction took place in these samples at a decreased rate. This would represent nonspecific inhibition of the enzyme reaction similar to the reduction of enzymatic hydrolysis rate at lower temperatures. If some specific interaction exists between certain phospholipids and the enzyme, it would likely involve those very tightly bound 3–4% of the total phospholipids that remain with the protein even after chromatography on Sepharose in 1% SDS (Peters et al., 1981b).

An alternative explanation could be that the decreased distance between individual enzyme molecular complexes could enable some long-range mutual inhibitory action.

DePont et al. (1978) showed that enzymatic conversion of phosphatidylserine to phosphatidylethanolamine of the (Na^+,K^+)-ATPase preparation had no effect on the

(Na^+,K^+)-ATPase activity. Further, they showed that enzyme hydrolysis of phosphatidylinositol had no effect on enzyme activity and that hydrolysis of PC, SM, and PE (by the same preparation of phospholipase C as used in this study) caused only 30–40% decrease in activity. They concluded there is no absolute requirement for any particular phospholipid polar head group for enzyme activity.

Hilden & Hokin (1976) have demonstrated that coupled Na^+–K^+ transport could be found in phospholipid vessels containing (Na^+,K^+)-ATPase with PC as the only phospholipid. Moore et al. (1978) found similar results with the Ca^{2+}-ATPase using diphenylhexatriene as a fluorescent probe of membrane fluidity and concluded that membrane fluidity is the rate-limiting factor of the Ca^{2+}-ATPase activity at physiological temperatures. This concept would also explain reported observations of Ahmed & Thomas (1971) that long-chain fatty acids have a inhibitory affect on the activity of the (Na^+,K^+)-ATPase presumably by decreasing the fluidity of the phospholipid bilayer.

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