# The Role of Lipid-Phase Transitions in the Regulation of the (Sodium + Potassium) Adenosine Triphosphatase†

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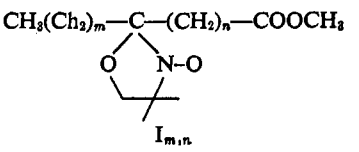
ABSTRACT: The (sodium + potassium) adenosine triphosphatase (($Na^+$ + $K^+$)-ATPase) purified from lamb kidney outer medulla undergoes a large change in activation energy near 20°. Above 20° the activation energy is 15.2 kcal/mol while below 20° it is 32.6 kcal/mol. The membrane lipids of the ATPase have been labeled with methyl 6-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)heptadecanoate. The order parameter for the label undergoes a sharp break at the same temperature as the change in activation energy for the ATPase. When the lipids are extracted from the membrane and labeled the transition is still observed, and so the change in enzyme activity that occurs at 20° is due to a change in state of the lipids. The temperature studies suggest that the ATPase must be in a "fluid-like" environment to function. When the purified membrane fragments containing the ATPase are compared with the crude plasma membrane fraction using a series of spin-labeled fatty acid esters it is found that the purified fragments are similar in the head-group region of the phospholipids but that the interior of the membrane is considerably more fluid, further supporting the suggestion that the membrane lipids must be fluid for the ATPase to function.

In bacteria there is a definite requirement for membrane fluidity for the function of membrane transport systems (Wilson and Fox, 1971). *Escherichia coli* and *Mycoplasma laidlawii* membranes undergo gel to liquid crystal-phase transitions which are dependent on the fatty acid composition of the membrane (Wilson and Fox, 1971; Steim *et al.*, 1969). Transport of β-galactosides occurs only when the membrane is in the liquid crystal state. The ($Na^+$ + $K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3), which is believed to be an integral part of the alkali cation transport system of higher animals, undergoes a change in activation energy near 20° (Gruener and Avi-Dor, 1966; Charnock *et al.*, 1971) which is similar to the temperature effects seen with the bacterial systems. Kimelberg and Papahadjopoulos (1972) observed that the ATPase inactivated with deoxycholate could be reactivated with dipalmitoylphosphatidylglycerol and that the change in activation energy occurred at 32° rather than 20°. Although they had not determined the phase transition temperature for dipalmitoylphosphatidylglycerol they suggested that the change in activation energy is due to a lipid-phase transition. In this paper we provide direct evidence that the change in activation energy is due to a lipid-phase transition.[1] It also appears likely that the phase transition affects enzyme activity by its effect on a conformational change in the protein.

## Experimental Section

*Materials.* The ($Na^+$ + $K^+$)-ATPase was purified from lamb kidney outer medulla according to the procedure of Grisham and Barnett (1972) and had a specific activity of 10–14 μmol of ATP hydrolyzed/mg of protein per minute. The plasma membrane fraction was prepared by the method of Schwartz *et al.* (1962). The fatty acid ester spin labels ($I_{m,n}$) were prepared by the methods of Waggoner *et al.* (1969).




*Methods.* The ATPase was assayed by the method of Barnett (1970). The purified ATPase and the plasma membrane fraction were spin labeled by the procedure of Grisham and Barnett (1972) with the modification that 0.003 M $MgCl_2$–0.10 M NaCl–0.010 M KCl–0.050 M imidazole (pH 7.0) was present. Electron paramagnetic resonance (epr) spectra were taken on a Varian EM500 epr spectrometer modified for variable temperature studies. Temperatures could be maintained to within 0.5°. The lipids were extracted from the purified ATPase by the method of Folch *et al.* (1957). The extracted lipids were dried as a thin film and resuspended in 0.05 M imidazole buffer (pH 7.0) by agitating with a Vortex mixer. The suspended lipids were spin labeled in the same manner as the purified ATPase.

## Results and Discussion

The temperature dependence of the activity of the ($Na^+$ + $K^+$)-ATPase in the presence of 0.003 M MgATP–0.10 N NaCl–0.010 M KCl–0.050 M imidazole (pH 7.0) is shown in Figure 1. A sharp change in activity occurs at 20°, confirming the results of Charnock *et al.* (1971). The activation energy above 20° is 15.2 kcal/mol while below 20° it is 32.6 kcal/mol. The values determined by Charnock *et al.* (1971) for the rabbit kidney enzyme are 13.5 and 28.5 kcal/mol.

When spin labels such as $I_{m,n}$ are incorporated into phospholipid bilayers and natural membranes they undergo anisotropic, axially symmetric motion (Hubbell and McConnell, 1971). An order parameter, *S*, can be calculated for the motion of the labels (Seelig, 1970). The epr spectrum of the purified ATPase labeled with $I_{10,4}$ is highly temperature de-

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[1] The term "phase transition" is being used in a purely phenomenological sense. It is possible that the changes in the membrane lipids sensed by the spin labels are "phase separations."

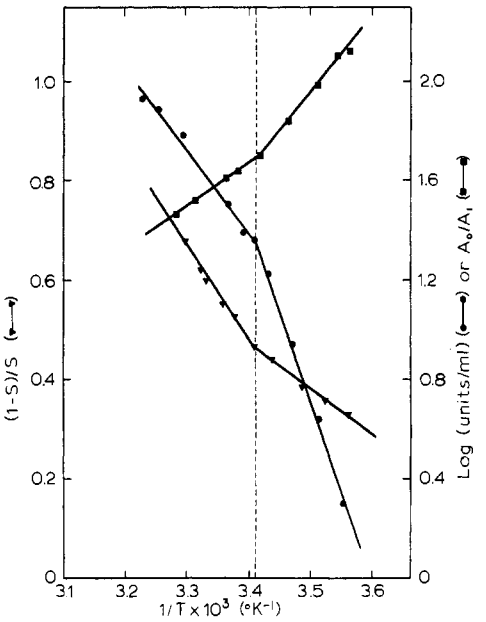


FIGURE 1: The temperature dependence of the activity of the ($Na^+$ + $K^+$)-ATPase in units per milliliter (●), of label $I_{10,4}$ in the purified ATPase membrane in terms of $(1 - S)/S$ (▼), and of label $I_{10,4}$ in the extracted lipids of the purified ATPase in terms of $A_0/A_1$ (■).

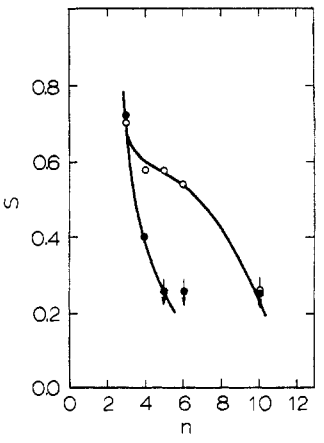


FIGURE 3: The dependence of the order parameter $S$ on $n$ for spin-labeled methyl stearates and heptadecanoates $I_{m,n}$ in the purified ATPase membrane (●) and in the crude plasma membrane (○) at 30°.

pendent (Figure 2). If $(1 - S)/S$ is plotted *vs.* $1/T$, two straight lines which intersect at 20°, the temperature of the change in enzyme activity, are obtained (Figure 1). When the extracted lipids are labeled with $I_{10,4}$, spectra similar to those obtained with the purified ATPase are obtained. However, the order parameter is smaller at all temperatures, indicating freer motion of the label, and so it could not be calculated accurately in the upper end of the temperature range. Instead, $A_0/A_1$, the ratio of the intensities of the middle and low field lines, respectively, has been plotted *vs.* $1/T$ (Figure 1). As with the intact ATPase two straight lines are obtained which intersect at 20°.

Since the extracted lipids exhibit a transition at the same temperature as the lipids of the intact membrane, the transition must be a characteristic of the lipids and not of the protein. The substantial change in activation energy that occurs in the activity of the ($Na^+$ + $K^+$)-ATPase at 20° must be due to a transition in the lipids. It is likely that the change in lipid structure reflected by the lipid spin label is due to a phase transition analogous to what occurs in the bacterial systems (Wilson and Fox, 1971; Steim *et al.*, 1969) or it is due to a phase separation (McConnell *et al.*, 1972). This strongly suggests that the membrane lipids must be fluid for the proper functioning of the ATPase and accounts for the great temperature sensitivity of both the ATPase and the alkali cation transport system.

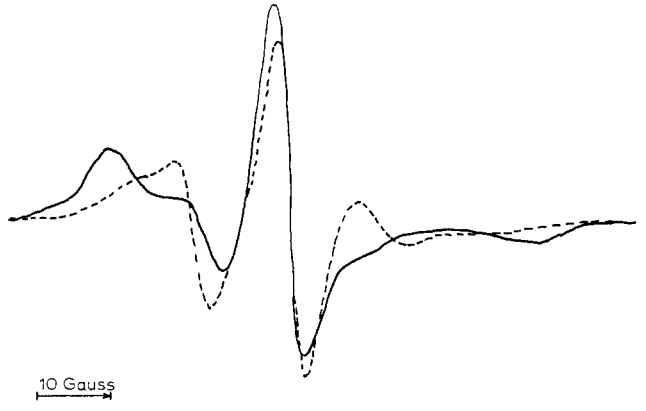


FIGURE 2: Epr spectra of label $I_{10,4}$ in the purified ATPase membrane at 5° (—) and at 32° (---).

Further support of the requirement for membrane fluidity in the functioning of the ($Na^+$ + $K^+$)-ATPase comes from studies with a series of stearate and heptadecanoate spin-labeled esters $I_{m,n}$ with $n$ = 3, 4, 5, 6, and 10. Both the purified ATPase and the crude plasma membrane fraction were labeled with the five spin-labeled fatty acid esters and the epr spectra were taken at 30°. The order parameters $S$ were calculated from the spectra and are shown in Figure 3. There is a striking difference between the two membrane preparations. With label $I_{12,3}$ the purified ATPase membrane and the crude plasma membrane appear similar. However, for the rest of the labels the interior of the purified ATPase membrane appears to be considerably more fluid than for the crude plasma membrane.

In the purified ATPase membrane the transition from a rigid environment to a fluid one occurs sharply on going from C-5 to C-6 of the spin-labeled fatty acid ester. This corresponds to going from $n$ = 3 to $n$ = 4 (Figure 3). The first double bond in arachidonic acid occurs between C-5 and C-6. Since this double bond is cis it produces a "kink" in the fatty acid chain. The correspondence between the point at which fluidity of the purified ATPase membrane becomes pronounced and the first double bond in arachidonic acid suggests that arachidonate side chains on the phospholipids may be responsible for the marked fluidity of the ATPase membrane in the vicinity of C-6. The comparison of the purified ATPase with the crude plasma membrane fraction is quite dramatic for $I_{10,4}$ as the probe (the spin label is at C-6). For label $I_{10,4}$ the purified ATPase membrane is more fluid at 10° than the crude plasma membrane is at 30°.

In qualitative terms the purified ATPase membrane is similar near the head-group region but more fluid in the interior than the crude plasma membrane. When this observation is coupled with the temperature dependence of the ATPase discussed above the case for a requirement on the part of the ($Na^+$ + $K^+$)-ATPase of the lipid fluidity is quite strong. Furthermore, the dependence of the ATPase on phase transitions in the lipids suggests that the ATPase and the alkali cation transport system *in vivo* may be regulated by

long-range cooperative interactions in the membrane lipids.

These results depend on how adequately the spin labels $I_{m,n}$ report the overall structure of the labeled membrane. If the labels were localized in small fluid pockets in the membrane one could be misled by the apparent fluidity reported by the labels. It is likely that the labels are reporting an overall structure of the membrane. We have previously shown (Barnett and Grisham, 1972) that the spin labels undergo rapid lateral diffusion in natural membranes and that there can be no substantial local pockets where the label is concentrated. The extent to which the labels themselves are perturbing the membrane structure cannot be answered at this time. However, the amount of label incorporated (*ca.* 1% of the total lipid present) is not large enough to have any effect on the activity of the ATPase.

The precise nature of the role of the lipid-phase transitions in regulating the activity of the $(Na^+ + K^+)$-ATPase has yet to be determined. However, a likely candidate for the step in the enzymatic sequence which is principally affected is the conformational change observed by Post *et al.* (1965).

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# Transbilayer Asymmetry and Surface Homogeneity of Mixed Phospholipids in Cosonicated Vesicles†

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ABSTRACT: Cosonication of equimolar quantities of phosphatidylglycerol (PG) and phosphatidylcholine (PC) results in bilayered vesicles the outer surface of which contain, on the average, twice as many PG as PC molecules. Within the surface these two lipid classes are not spatially segregated into "patches." These results were obtained by exploiting the effects of paramagnetic ions on the proton and phosphorus nuclear magnetic resonances. The $^{31}P$ resonances of PG and PC sonicated separately have different chemical shifts and broaden differently upon addition of $Mn^{2+}$. At $Mn^{2+}$ concentrations less than $10^{-4}$ M, these ions do not permeate the vesicles, permitting a distinction of the signals originating on the outer surface from those on the inner surface. For pure dispersions of PG and of PC, $Mn^{2+}$ and $Eu^{3+}$ reside closer to the phosphate than to the choline $N(CH_3)_3^+$ protons and the residence time of $Mn^{2+}$ is short, less than $10^{-4}$ sec. The integrated and asymmetric arrangement of the phospholipid molecules in the cosonicated dispersions is discussed in the context of the structure and biosynthesis of biological membranes.

Phospholipids are a major constituent of many cellular membranes. These molecules are present in several classes, each defined according to its polar head group, and within each class there is great diversity in the fatty acid composition. The variability in the fatty acid composition has been investigated extensively, and its physiological significance is beginning to be understood (Cronan and Vagelos, 1972; Singer and Nicolson, 1972); however, little is known about the function of the variability in the head-group composition. Nevertheless the observations that different membranes often contain different phospholipid classes (Rouser *et al.*, 1968; Kates and Wassef, 1970) and that in some cases cells alter their membrane phospholipid composition in response to changes in external parameters (Op den Kamp *et al.*, 1969, 1971; Haest *et al.*, 1972) suggest that this variability is of structural and physiological importance.

The majority of the phospholipids are believed to be ar-

† From the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received March 26, 1973.* This is the sixth paper in the series: Magnetic Resonance Studies of Membrane and Model Membrane Systems. A preliminary account of this work was presented at the International Conference on ESR and NMR in Biology and the 5th Conference on Magnetic Resonance in Biological Systems, New York Academy of Science, Dec 1972, and at the U.C.L.A. Conference on Membranes, Squaw Valley, Calif., March 1973. This work was supported by the U. S. Atomic Energy Commission.

‡ This material will be submitted in partial fulfillment of the Ph.D. requirement of the University of California, Berkeley.