Role of Phospholipids in the Calcium-dependent ATPase of the Sarcoplasmic Reticulum

ENZYMATIC AND ESR STUDIES WITH PHOSPHOLIPID-REPLACED MEMBRANES*

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Three types of partially purified ATPase enzymes having different phospholipid contents and compositions have been prepared: (a) an enzyme whose phospholipid moiety has been replaced predominantly by dioleoyl lecithin (DOL-enzyme), with about the same phospholipid content as the original sarcoplasmic reticulum, (b) dipalmitoyl lecithin-replaced enzyme whose phospholipid content is 30% of that of DOL-enzyme (DPL-enzyme), and (c) a partially delipidated enzyme with about the same phospholipid content as DPL-enzyme but with the original sarcoplasmic reticulum phospholipid composition (del-enzyme). The temperature dependence of Ca**-activated ATPase activity of these preparations showed clearcut differences; with DOL-enzyme there was no appreciable break in the Arrhenius plot in the 3-40° range; DPL-enzyme showed a break at 29°, and del-enzyme and sarcoplasmic reticulum one at 18°. Transition temperatures obtained from ESR studies with the use of spin-labeled stearic acid incorporated into the membranes agreed with those derived from ATPase assays. Thermodynamic analysis of the ATP hydrolysis rates shows that DPL-enzyme has considerably larger values of activation enthalpy and activation entropy below the transition temperature (29°) than those of the other preparations, while all enzyme preparations show similar free energies of activation. The ESR data show that below their transition temperatures DPL-enzyme, and to a lesser degree del-enzyme, have a strongly restricted motion of their phospholipid molecules as compared with either DOL-enzyme or sarcoplasmic reticulum.

Studies on the formation and decomposition of phosphoenzyme have been carried out with the three types of ATPase preparations. At 0°, the rate of inorganic phosphate liberation is 8 times lower in DPL-enzyme than in del-enzyme with little difference in the steady state level of phosphoenzyme. In DOL-enzyme, the level of phosphoenzyme and the rate of inorganic phosphate liberation are 1.8 and 3.5 times higher than the corresponding values obtained with del-enzyme. Addition of ADP to the phosphorylated intermediate of DPL-enzyme induces a fast reversal of the phosphorylation reaction. These results indicate that the physical state of the phospholipid molecules associated with the enzyme affects the decomposition of phosphoenzyme, with little effect on the phosphorylation reaction and its reversal.

Phospholipids are essential components of the Ca**-dependent ATPase activity in the sarcoplasmic reticulum. Removal of the membrane-associated phospholipids by means of phospholipase digestion leads to loss of the ATPase activity, while readdition of phospholipids restores it (1). Recent reports that Ca**-accumulating vesicles can be reconstituted from the purified ATPase enzyme and phospholipids (2-4) suggest that they are also essential for the Ca** accumulation by the SR vesicles.

In the last few years some insight has been gained into the mechanism by which phospholipids affect the ATPase reaction and the coupled Ca** transport. ESR studies using spin-labeled analogues of stearic acid and spin probes attached to the ATPase protein have shown that ATPase activity and Ca** transport are related to the fluidity of the membrane lipids, reflected in the mobility of spin labels. Restriction of the

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mation of the phospholipid molecules by reducing the temperature inhibits ATPase activity (5). Delipidation also produces a substantial decrease in label mobility and a strong inactivation of ATPase activity; upon readdition of lipids these effects are reversed (6).

A considerable body of evidence has accumulated indicating the existence of various states of the ATPase enzyme, including one or more phosphorylated forms, in the course of the hydrolysis of ATP. In SR vesicles delipidated by means of digestion with phospholipases, both the rate and level of phosphoenzyme formation are about the same as in the intact SR, while the steady state P\textsubscript{i} liberation is considerably inhibited (1, 7). This suggests the delipidation primarily affects the decomposition of the phosphoenzyme (for an opposing view, see Ref 8).

Recently methods have been developed for the replacement of the endogenous phospholipids of the purified ATPase with various synthetic lecithins (4). It has been shown that replacement with dipalmitoyl lecithin and dimyristoyl lecithin—phosphatidyl choline that contain saturated hydrocarbon chains—completely inhibits ATPase activity at lower temperatures, while further replacement with dioleoyl lecithin, a phospholipid that contains an unsaturated fatty acid moiety, releases the inhibition of ATPase at low temperatures (9).

The present studies have been carried out in an attempt to further clarify the relation of physical properties of the membrane and certain aspects of the ATPase reaction to the structure of the fatty acid moiety of the phospholipids. From parallel studies of ATPase activity and mobility of spin-labeled stearic acids incorporated into ATPase enzymes containing predominantly either DOL or DPL, it appears that decreased fluidity of the lipid moiety decreases the ATPase activity. Thermodynamic analysis indicates that low fluidity makes the transition associated with activation energetically unfavorable, which is reflected in higher activation enthalpy, at the same time that it imposes "order" on the enzyme, which is reflected in higher activation entropy. Studies on the formation and decomposition of the phosphorylated intermediate show that phospholipids affect primarily the dephosphorylation process. Thus replacement with DPL results in a strong inhibition of the rate of ATP hydrolysis at temperatures at which the mobility of the DPL molecules is strongly restricted, while there is only a small effect on the phosphorylation reaction. These results have confirmed and extended previous suggestions, originated from the results obtained with phospholipase-digested SR, concerning selective involvement of SR lipids in the decomposition of phosphoenzyme during the ATPase reaction.

**EXPERIMENTAL PROCEDURE**

Replacement of Endogenous Phospholipids Fragmented SR was prepared from rabbit white skeletal muscle as reported previously (10). For the replacement of phospholipids the original method as described by Warren et al. (4) was modified as follows. Freshly prepared SR (25 to 60 mg of protein) was incubated with slow stirring at 0\textdegree C for 2 hours with a sonicated mixture of deoxycholate and phospholipid in 3 to 6 ml of a solution containing 1 mM KCl, 0.3 mM sucrose, 10 mM \beta-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0. The weight ratio of protein, deoxycholate and phospholipid used was 1:1:1 for replacement by DOL, and 1:0.3:2 for replacement by DPL. One milliliter of the resulting clear solutions was placed on top of a discontinuous sucrose gradient obtained by layering 2 ml of 15% (w/v) sucrose on 2 ml of 50% sucrose; both sucrose solutions contained 1 mM KCl, 0.5 mM Tris-HCl, pH 8.0, and 10 mM \beta-mercaptoethanol. The tubes were centrifuged for 17 hours at 5\textdegree C in a Beckman SW 50.1 rotor at 150,000 \times g. Both phospholipid-replaced preparations formed turbid bands, DOL-enzyme at the interface of the two sucrose layers and DPL-enzyme in the 50% sucrose layer, between the upper and middle third. After centrifugation from the bottom of the tubes, the phospholipid-replaced preparations were diluted 5-fold with 50 mM Tris-maleate, pH 7.0, and were centrifuged at 4\textdegree C for 60 min at 100,000 \times g. The resulting pellets were homogenized in a solution of 0.3 mM sucrose/20 mM Tris-maleate, pH 7.0, at 0\textdegree C, to a final protein concentration of 4 to 6 mg/ml. Less than 10 \mu g of deoxycholate/mg of protein, as determined with the use of [\textsuperscript{14}C]deoxycholate, remained in all replaced preparations.

The single step procedure for phospholipid replacement described here, in which SR instead of purified enzyme was used as the starting material for replacement, resulted in a concomitant purification of the ATPase; examination of the phospholipid replaced membranes by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate showed that they contained essentially a single 100,000-dalton band. The ATPase activity was stable for at least 3 days. Deoxycholate and DPL were obtained from Sigma Chemical Co., and DOL from Scientific Products. Deoxycholate was recrystallized prior to use; both DPL and DOL were used without further purification.

Measurement of ATPase Activity—Reaction mixtures contained 0.1 M KCl, 5 mM MgCl\textsubscript{2}, 0.6 mM EGTA, 0.5 mM CaCl\textsubscript{2}, 2 mM ATP, 50 mM Tris-maleate, pH 7.0, and protein as stated in the figure legends. To obtain the Ca\textsuperscript{2+}-dependent ATPase activity, the basal activity determined in the above medium plus 5 mM EGTA was subtracted. The amount of inorganic phosphate liberated was determined by the method of Fiske-SubbaRow (11) and the concentration of protein according to Lowry et al. (12) using bovine serum albumin as standard. Incubation times up to 130 min on 50% sucrose layer, between the upper and middle third. After centrifugation from the bottom of the tubes, the phospholipid-replaced preparations were diluted 5-fold with 50 mM Tris-maleate, pH 7.0, and were centrifuged at 4\textdegree C for 60 min at 100,000 \times g. The resulting pellets were homogenized in a solution of 0.3 mM sucrose/20 mM Tris-maleate, pH 7.0, at 0\textdegree C, to a final protein concentration of 4 to 6 mg/ml. Less than 10 \mu g of deoxycholate/mg of protein, as determined with the use of [\textsuperscript{14}C]deoxycholate, remained in all replaced preparations.

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**Formation and Decomposition of Phosphoenzyme**—The reaction mixture contained 0.1 M KCl, 5 mM MgCl\textsubscript{2}, and 20 mM Tris-maleate, pH 6.5. Concentrations of Ca\textsuperscript{2+}, EGTA, [\textsuperscript{32}P]ATP, ADP, and ATP are indicated in the legends to figures and tables. For measurement of phosphoenzyme formation, the reaction was stopped by adding trichloroacetic acid to a final concentration of 6.7%; 0.5-ml fractions were filtrated through Millipore filters (HA, 0.45 \mu m pore size) previously washed with 1 ml of 0.1 mM ATP to prevent binding of ATP to the filter. The acid-denatured protein retained on the filter was washed with 5-ml portions of a solution containing 5% trichloroacetic acid and 0.2 mM NaH\textsubscript{2}PO\textsubscript{4}. The filters were dried and counted in a liquid scintillation counter in vials containing 2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl)-toluene solutions. To determine the amount of inorganic phosphate liberated, centrifuged portions of the reaction mixture were treated with activated charcoal to remove the unreacted [\textsuperscript{32}P]ATP, followed by filtration through surgical cotton. Portions (0.2 ml) of the filtrate were placed on filter paper strips, which were then dried and counted. [\textsuperscript{32}P]ATP was prepared according to Glynn and Chappell (13) with the use of [\textsuperscript{32}P]orthophosphate obtained from New England Nuclear Corp.
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after hydrolysis to inorganic phosphate (15). To resolve the individual phospholipid components the extract was subjected to two-dimensional thin layer chromatography on silica gel plates (Merck, 250 μ thickness). A mixture of chloroform, methanol, concentrated ammonia, and water (80/80/2.7/7.3) was used for development in the first dimension, and a mixture of chloroform, methanol, acetone, glacial acetic acid, and water (90/10/10/10) for development in the second dimension. The individual phospholipid spots were located by exposing the plates to iodine vapor. After scraping the spots from the plates their phospholipid content was determined. A factor of 22.5 was used to convert milligrams of phosphate to milligrams of phospholipid. Fatty acid compositions of phospholipid replaced preparations and SR were determined by gas-liquid chromatography. The fatty acids were transesterified to their methyl esters and analyzed in a Varian model 2700 gas liquid chromatographic apparatus.

ESR measurements—N-Oxyl-4',4'-dimethyloxazolidine derivatives of stearic acid having the general formula \( \text{C}_n\text{H}_{2n+1}\text{O}_{2n+2} \) were used as spin probes. They were dissolved in benzene at a concentration of 10 mM and stored at -20°C.

Aqueous solutions of the labels were prepared by evaporating to dryness a small amount of the benzene solution prior to dissolving it in 0.025 ml of methanol to which 0.5 ml of 0.3 M sucrose/20 mM Tris-maleate, pH 7.0, was added. For incorporation of spin labels into membranes, the spin label solution was mixed with the membrane suspension—the final concentration of methanol was less than 0.5%—by vigorous shaking at room temperature. Final concentration of protein was 5 to 10 mg/ml. The spin label was added in ratios ranging from 0.4 to 1.7 μmol/100 mg of protein. These values correspond to 0.6 to 2.6 spin labels per 100 molecules of membrane lipid for SR and DOL-enzyme and 2 to 9 spin labels per 100 molecules of lipid in the delipidated preparations, viz. del-enzyme and DPL-enzyme. Similar spin label/lipid ratios have been used by other workers (6). There were no noticeable differences in the spectra in the range of spin label ratios used.

Three different methods were used to interpret the recorded ESR spectra. For spectra that indicated strongly hindered motion of the spin label, the splitting between the low and high field extrema (2Tc) was used as an indication of immobilization. With other preparations it was feasible to calculate the order parameter, S, introduced for fatty acid spin labels undergoing anisotropic motion around their long molecular axis, as reported previously (16).

For the spin label 1(1,14) spectra were evaluated in terms of an empirical motion parameter for nearly isotropic motion, τ (17):

\[
\tau = 6.5 \times 10^{-19} W_0 \left( \frac{h_x}{h_z} \right)^{1/2} \left( \frac{h_z}{h_x} \right)^{1/2} W_0
\]

where \( W_0 \) is the line width of the midfield line and \( h_x \) and \( h_z \) are the heights of the mid and high field lines of the spectrum, respectively (see Fig. 7). Greater freedom of motion is associated with smaller values of either 2Tc, S, or τ. All ESR spectra were recorded using a Varian-4052 spectrometer equipped with a temperature controlling unit. The spin-labeled stearic acid derivatives were purchased from Synva Co.

RESULTS

Phospholipid Content and Composition after Replacement—Table I shows the phospholipid content of several types of enzyme preparations and SR. DOL-enzyme has the same amount of phospholipid as SR, whereas the phospholipid content of DPL-enzyme is reduced by 70%. By using the procedure described for phospholipid replacement and varying the deoxycholate to protein ratio without adding exogenous phospholipids, preparations with different phospholipid content can be obtained. The higher the ratio of deoxycholate to protein, the lower the amount of phospholipid remaining associated with the protein. To obtain an enzyme preparation with the same phospholipid content as DPL-enzyme, 0.75 mg of deoxycholate/mg of protein were used. The resulting preparation (del-enzyme, Table I, has the same phospholipid composition as the original SR, which differs significantly from the phospholipid composition of DOL-enzyme and DPL-enzyme (Table III). In agreement with previous reports (7, 18) five different phospholipid species are present in SR, of which phosphatidyl choline amounts to 67% of the total. On the other hand, both DPL-enzyme and DOL-enzyme contain more than 90% of phosphatidyl choline. The results of the fatty acid analysis show that DPL-enzyme contains mainly saturated fatty acids, of which palmitic acid (16:0) represents 85%. In contrast, the DOL-enzyme system contains mostly unsaturated fatty acids of which oleic acid (18:1) accounts for about 85%.

Temperature Dependence of Ca2+-ATPase Activity—A sharp break occurs at 29°C in the Arrhenius plot of DPL-enzyme indicating a steep drop in Ca2+-ATPase activity below that temperature (Fig. 1). The plots for SR, in agreement with previous results (5, 19), and of del-enzyme show a less steep bend at 18°C. In the case of DOL enzyme there is no break in the Arrhenius plot in the temperature range studied (3–40°C); this absence of a transition temperature might be attributed to the fact that the phase transition for pure DOL occurs below 0°C (20). The activation energies and the transition temperatures of the Ca2+-ATPase activity for these different preparations are summarized in Table III. Below the transition temperature the

| Table I | Total phospholipid content of various ATPase preparations |
|---------|-----------------------------------------------------------|
| Preparations | Phospholipid content (mg phospholipid/mg protein) |
| SR | 0.45 ± 0.06 (4) |
| del-enzyme | 0.12 ± 0.02 (3) |
| DOL-enzyme | 0.46 ± 0.08 (2) |
| DPL-enzyme | 0.14 ± 0.02 (11) |

| Table II | Phospholipid and fatty acid composition of various ATPase preparations |
|--------|----------------------------------------------------------------|
| Composition | SR | del-enzyme | DOL-enzyme | DPL-enzyme |
| Phospholipid (%) | PC | 65 | 63.9 | 92.4 | 91.0 |
| PE | 20.6 | 19.3 | 5.1 | 5.5 |
| PI | 9.2 | 6.7 | 2.5 | 3.5 |
| PS | 2.9 | 3.8 | tr | tr |
| SM | 2.3 | 0.3 | tr | tr |
| Fatty acid (%) | 14:0 | 0.3 | tr | 0 |
| 16:0 | 42.1 | 8.5 | 85.5 |
| 18:0 | 12.3 | 2.5 | 7.6 |
| 18:1 | 18.8 | 84.7 | 7.4 |
| 18:2 | 26.6 | 4.1 | tr |
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activation energy for DPL-enzyme, 43.5 kcal/mol, is considerably higher than those of the other preparations, which fall in the range of 30 kcal/mol below their transition temperatures. Above the transition temperatures, however, all activation energies are similar and in the range of 20 to 25 kcal/mol. Although del-enzyme has the same transition temperature as SR, the ATPase rate of the former has a higher activation energy, suggesting that the considerably reduced lipid content of del-enzyme might have some effect on the enzyme reaction rates. As shown in Table III, the differences in activation energy are compensated by differences in entropy of activation, resulting in values of free energy of activation roughly independent of variations in temperature, phospholipid content and composition (16 to 18 kcal/mol).

Membrane Fluidity as a Function of Temperature—The ESR spectra of different preparations labeled with I(5,10) at various temperatures fell into two categories (Fig. 2). Spin-labeled SR (Fig. 2A) and DOL-enzyme (Fig. 2B) have spectra that reflect a label which changes from a fairly immobilized state at low temperatures (e.g. 3.5°) to an almost isotropic motion around 25°. In contrast, the spectra obtained with labeled DPL-enzyme and del-enzyme indicate a strongly immobilized label in the whole temperature range studied. Semilogarithmic plots of 2T′ (Fig. 3A) or of (1-S)/S (Fig. 3B) versus 1/T show breaks at 17° in the case of SR labeled with I(5,10) but no discernible break in the case of DOL-enzyme. The same type of plots for I(5,10)-labeled del-enzyme and DPL-enzyme show breaks at 18° and 29°, respectively (Fig. 4). Also the slopes are considerably steeper in the case of SR and DOL-enzyme than for DPL-enzyme and del-enzyme.

Fig. 5 shows the ESR spectra of DPL-enzyme and del-enzyme obtained with I(1,14), which has the nitroxide label at a more distal position from the polar group. Increasing the temperature in this case produces a much larger increase in label mobility than observed with I(5,10) (cf. Fig. 2). Because of the increased mobility of the label, the parameter T (see "Experimental Procedure") was more appropriate for the analysis of the spectra. A semilogarithmic plot of T versus 1/T (Fig. 6) also shows breaks at 29° and 18° for DPL-enzyme and del-enzyme, respectively. In contrast to I(5,10), with both preparations showed similar values of 2T′ at all temperatures, labeling with I(1,14) results in significantly higher values of T for DPL-enzyme. This would indicate that the segments of the alkyl chains near the methyl ends are in a more fluid environment in del-enzyme than in DPL-enzyme. The differences become smaller at higher temperatures.

Effect of Phospholipid Replacement on Formation and Decomposition of Phosphoenzyme— Upon addition of ATP at 0°, the phosphorylated intermediate of del-enzyme reaches its maximum level in 8 s and it remains constant during the steady state of inorganic phosphate liberation (Fig. 7A). A similar feature has been observed with SR (cf. Ref. 21). The formation of phosphoenzyme with DPL-enzyme occurs in two steps; a rapid rise of phosphoenzyme in the first few seconds, followed by a gradual increase to reach in 120 s comparable values to the steady state levels obtained with del-enzyme. In contrast to this similarity between both systems with respect to phosphoenzyme levels, the steady state rate of inorganic phosphate liberation is much smaller in DPL-enzyme than in del-enzyme, with average rates of 0.30 and 2.40 mmol of Pi/mg protein/min, respectively (Table IV). The phosphoenzyme level and the rate of inorganic phosphate liberation in DOL-enzyme are 1.8 times (cf. Fig. 7, A and B) and 3.5 times (Table IV) larger than the respective values of del-enzyme. The corresponding rate constants (viz. rate of Pi liberation/phosphoenzyme) during the steady state is 0.89 min⁻¹ for del-enzyme and 1.76 min⁻¹ for DOL-enzyme. In the case of DPL-enzyme the rate constants are 0.16 min⁻¹ in the early phase (4 s) and 0.12 min⁻¹ in the late phase of the reaction (120 s). The significantly lower rate constants with DPL-enzyme indicate a strong inhibition of phosphoenzyme decomposition.
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Fig. 2. ESR spectra obtained at various temperatures of I(5, 10) incorporated into membrane preparations. A, SR; B, DOL-enzyme; C, DPL-enzyme; D, del-enzyme. The horizontal bar represents 10 Gauss.

Fig. 3. A, semilogarithmic plot of $2T_1$ versus $1/T$. ▼, SR and ●, DOL-enzyme. Spin label: I(5,10). B, Semilogarithmic plot of $(1 - S)/S$ versus $UT$. ▼, SR and ●, DOL-enzyme. Spin label: I(5,10). The order parameter $S$ was calculated from the spectra as described elsewhere (16).

In order to further characterize the kinetics of DPL enzyme, we have studied in this system the reversal of the phosphorylation reaction. It has been demonstrated in the intact SR vesicles that ATP can be synthesized from ADP and inorganic phosphate (22). Furthermore, during the course of the ATPase reaction either with SR (21) or with the purified CA$^+$-ATPase enzyme (23), addition of ADP results in the resynthesis of ATP at the expense of phosphoenzyme, with no appreciable changes.
in the amount of inorganic phosphate liberated. In the experiment shown in Fig. 8, 0.2 mM ADP was added to DPL-enzyme at 4 s or 90 s after starting the reaction with 5 μM ATP. In either case the addition of ADP induced a rapid decay of phosphoenzyme to the level obtained when the reaction was started with 5 μM ATP in the presence of 0.2 mM ADP. There were no appreciable changes in phosphate levels on addition of ADP. These results indicate that the mechanism responsible for the strong inhibition of inorganic phosphate liberation in DPL-enzyme does not interfere with the reversal of the phosphorylation reaction. It also appears that the phosphorylated intermediate of DPL-enzyme is readily accessible to ADP for the reverse reaction in the early and late phases of reaction.

**DISCUSSION**

The current view of the mechanism of the ATPase reaction coupled with Ca²⁺ transport in sarcoplasmic reticulum is summarized in the following set of equations (24, 25):

\[
E + n\text{Ca}^{2+} \rightleftharpoons E(\text{Ca})_n \quad (5)
\]

\[
E(\text{Ca})_n + \text{ATP} \rightleftharpoons PE(\text{Ca})_n + \text{ADP} \quad (6)
\]

\[
PE(\text{Ca})_n \rightleftharpoons PE^*(\text{Ca})_n \quad (7)
\]

The initial step of the reaction (Equation 5) is the binding of Ca²⁺ to an n number of high affinity sites (viz. α sites) on the enzyme molecule (26, 27). In Equation 6 the enzyme is phosphorylated by the γ-phosphate of ATP (21, 28), and in Equation 7 the enzyme changes to a new conformation in which the α site has a low affinity for Ca²⁺ (27). Ca²⁺ is released from the enzyme in Equation 8 before dephosphorylation of the enzyme takes place (Equation 9 and Ref. 24). A recent report by Froelich and Taylor (29) suggests the existence of an acid-labile form of phosphoenzyme (E·P⁴) in the ATPase reaction, which would be tentatively identified with PE⁺(Mg) on Equations 8 and 9.

There is some controversy concerning the role phospholipids play in these intermediate reaction steps. According to Fiehn and Hasselbach (8), delipidation of the SR vesicles by means of digestion with phospholipase A, followed by treatment with...
The recent method of Warren et al. (4), which permits replacement of the endogenous phospholipids present in SR by various exogenously added phospholipids, has various advantages over enzymatic delipidation. Since the ATPase protein is stable, pH 6.5. The reaction was started by adding the enzyme preparations (0.2 mg protein/ml) and it was stopped by adding 6.7% trichloroacetic acid at the times indicated on the abscissa. Phosphoenzyme activity is in parentheses.

| Preparation | Initial rate | % |
|-------------|-------------|---|
| DOL-enzyme  | 8.40 ± 1.20 | 100 |
| del-enzyme  | 2.40 ± 0.60 | 28.6 |
| DPL-enzyme  | 0.30 ± 0.06 | 3.6 |

bovine serum albumin, results in a strong inhibition of ATPase activity, with considerable reduction in the levels of phosphoenzyme formation. This could mean that phospholipids might be required in the phosphorylation step (Equation 6). On the other hand, SR preparations delipidated with various phospholipases (1, 7), while having a strongly inhibited ATPase activity, are phosphorylated by ATP to nearly the same extent as the intact membrane. These results have been interpreted in terms of a selective involvement of phospholipids in the steps leading to phosphoenzyme decomposition. A recent kinetic study on delipidated SR (30) lends support to this view.

The discrepancy in the effect of phospholipids on the stability of the phosphoenzyme may be ascribable, at least partially, to several problems inherent in earlier techniques of delipidation. For instance, it is rather difficult to control the extent of delipidation by lipase digestion, since a considerable fraction of the total phospholipids (viz. about 20% of the original content, Ref. 7) remains undigested and attempts to remove all phospholipids often result in an irreversible inactivation of the enzyme. Furthermore, digestion products, whose nature depends on the species of lipase used, would induce various modifications of the properties of the membrane (1, 7, 8).

A modified version of the replacement technique, which has the added advantage of allowing phospholipid replacement and partial purification of the ATPase enzyme at the same time, has been used in the present study with the aim of investigating the effect of phospholipid replacement by well defined phospholipid molecules—DPL and DOL—or the partial kinetic steps of the ATPase reaction. Replacement by DOL yields an enzyme preparation in which steady state levels of phosphoenzyme (at 0') are similar to those obtained with our conventional preparations of purified ATPase (27), but the rates of P, liberation are decreased by 25%. In contrast, replacement by DPL produces at 0° a very large inhibition of P, liberation, with little effect on phosphoenzyme formation. This inhibition is due, to a large extent, to the replacement of the original phospholipids by DPL and not to the accompanying delipidation produced during replacement, since the phosphate liberation rates in DPL-enzyme are about 8 times lower.

FIG. 7. A, phosphoenzyme formation and P, liberation at 0° with DPL-enzyme and del-enzyme. The reaction mixture contained 0.1 M KCl, 5 mM MgCl₂, 0.6 mM EGTA, 0.5 mM CaCl₂, 5 μM [γ-32P]ATP, and 20 mM Tris-maleate, pH 6.5. The reaction was started by adding the enzyme preparations (0.2 mg protein/ml) and it was stopped by adding 6.7% trichloroacetic acid at the times indicated on the abscissa. Phosphoenzyme and P, were determined as described under "Experimental Procedure." •, O, DPL-enzyme; □, △, del-enzyme. Filled symbols, phosphoenzyme; open symbols, P,. B, Formation of phosphoenzyme and P, liberation for DOL-enzyme at 0°. Experimental conditions were as in A. ▲, phosphoenzyme; Δ, P,.

FIG. 8. Effect of ADP on phosphoenzyme formation and P, liberation by DPL-enzyme at 0°. The reaction mixture contained 5 μM ATP, 0.6 mm EGTA, 0.5 mM CaCl₂, and 0.2 mg/ml of protein; other conditions were as described in the text. The reaction was started with DPL-enzyme; ADP, to give a final concentration of 0.2 mM, was added at 0, 4, or 90 s after the start of the reaction. To stop the reaction 6.7% trichloroacetic acid was added at the times indicated in the abscissa. •, O, no ADP added; □, △, ADP added from the start of the reaction; ▲, ADP added at 4 s; ▲, △, ADP added at 90 s. Filled symbols, phosphoenzyme; open symbols, P,.
from the ESR spectra (cf. Table V). A similar result has already been reported for native SR (5). Our studies utilizing derived from enzymatic assays are identical with those derived present we cannot explain the reasons for these discrepancies.

The ESR spectra of DPL-enzyme indicate the strongest spin label immobilization below the transition temperature. This finding suggests that the lipid phase in DPL-enzyme is in a highly ordered state in this temperature range, which presumably reflect membrane fluidity and ATPase activity. Thus SR and del-enzyme, which differ considerably in their total phospholipid content but show similar phospholipid composition, exhibit the same transition temperature (cf. Tables I and V). On the other hand, replacement by either DOL or DPL, whose transition temperatures are -20 and 42°C (20) respectively, leads to large changes in the transition temperature of the corresponding enzyme systems. In DOL-enzyme there is no discernible transition temperature in the 3-40°C range, suggesting that DOL substitution shifts the transition temperature to a value below 3°C, while in the DPL-replaced system the transition temperature is increased from 18°C to 29°C. It should be pointed out that, while in our experiments the break in ATPase plots occurs at the same temperature as in ESR-mobility plots, comparison of preparations that have been subjected to different treatments (DOL enzyme versus SR) fails to reveal a correlation between absolute values of ATPase activity and spin label mobility.

The existence of a transition temperature, reflected in some parameters of membrane fluidity, is generally attributed to a phase transition from the crystalline to the liquid-crystalline state of the phospholipid moiety (2, 31, 32). However, the fact that in DPL-enzyme the transition temperature differs appreciably from the one obtained with pure DPL may indicate that lipid-protein interactions are also involved in the regulation of membrane fluidity and ATPase activity. Assuming that lipid-protein interactions modify the transition temperature of the lipid phase, this result could still be attributed to a lipid phase transition. Nevertheless, the possibility that lateral phase separations or cluster formations in the lipids are instrumental in producing the transition temperature cannot be excluded.

While this work was in progress, Warren et al. (9) described the effect of lipid replacement by DPL, DML, and DOL on the ATPase activity of purified enzyme preparations derived from SR. Their results show that in the case of the saturated lecithins, viz. DPL and DML, complete inhibition of ATPase activity takes place below 28°C and 24°C, respectively, and that no phosphorylation of the enzyme takes place below these temperatures. These results are at variance with our findings, since with DPL-enzyme we could detect ATPase activity even at 0°C. Furthermore, the phosphorylation reaction at this temperature was virtually unaffected by DPL-replacement. At present we cannot explain the reasons for these discrepancies.

As shown in this paper, the transitional temperatures derived from enzymatic assays are identical with those derived from the ESR spectra (cf. Table V). A similar result has already been reported for native SR (5). Our studies utilizing partially purified ATPase containing well defined phospholipid species show that the phospholipid composition seems to be a crucial factor controlling the transition temperature of both ESR spectra, which presumably reflect membrane fluidity and ATPase activity. Thus SR and del-enzyme, which differ considerably in their total phospholipid content but show similar phospholipid composition, exhibit the same transition temperature (cf. Tables I and V). On the other hand, replacement by either DOL or DPL, whose transition temperatures are -20 and 42°C (20) respectively, leads to large changes in the transition temperature of the corresponding enzyme systems. In DOL-enzyme there is no discernible transition temperature in the 3-40°C range, suggesting that DOL substitution shifts the transition temperature to a value below 3°C, while in the DPL-replaced system the transition temperature is increased from 18°C to 29°C. It should be pointed out that, while in our experiments the break in ATPase plots occurs at the same temperature as in ESR-mobility plots, comparison of preparations that have been subjected to different treatments (DOL enzyme versus SR) fails to reveal a correlation between absolute values of ATPase activity and spin label mobility.

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Recently, Lee et al. (33) have shown that a complex of purified ATPase and DOL exhibits a transition temperature at 29°C in terms of its ATPase activity. They attribute this result to the presence of quasicrystalline clusters in the DOL moiety, since they find the same transition temperature in the partition of the spin probe TEMPO incorporated into DOL bilayers. It is difficult to reconcile these results with our finding that in DOL-enzyme, both by ESR and ATPase measurements, no transition temperature is discernible in the 3-40°C range. Also worth noting in regard to DOL-enzyme is the fact that while below 17°C ESR studies show similar spin label mobilities for this system and native SR, above this temperature a significantly steeper increase in label mobility was found for SR. Although the DOL molecules in DOL-enzyme are most likely to be already in the liquid-crystalline state at 17°C, the lipids of SR include several types of unsaturated fatty acid molecules with multiple double bonds (9), which above the average transition temperature would produce a much more fluid membrane than DOL, which has only one double bond in each hydrocarbon chain.

The ESR spectra of DPL-enzyme indicate the strongest spin label immobilization below the transition temperature. This finding suggests that the lipid phase in DPL-enzyme is in a highly ordered state in this temperature range, which might be expected for a system whose predominant lipid moiety contains two saturated hydrocarbon chains. However, on the basis of the greater label immobilization in del-enzyme than in intact SR, some of the immobilization in DPL-enzyme might be attributable to its reduced phospholipid content. The

| Measured variable | Transition temperatures, °C |
|-------------------|----------------------------|
|                   | SR | DOL-enzyme | del-enzyme | DPL-enzyme |
| Ca2+ ATPase       | 18 | 18          | 29          | 29          |
| (1 - S)/S, l(5,10)| 17 | 17          | 29          | 29          |
| r E(1,14)         | 18 | 18          | 29          | 29          |

a*: no transition temperature was found in the range of temperatures studied.

TABLE V

Transition temperatures for various preparations calculated from Ca2+ ATPase activity and ESR measurements

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would affect the protein in the same way as lipid replacement producing an increased AS$ while also somehow energetically between DPL-enzyme and SR can be similarly explained: the rate limiting step(s), suggests that the increased order of the phosphoenzyme. The fact that both AH$ and ASS are than the bulk lipid in the membrane (9,34).

The thermodynamic parameters of activation are those of the rate-limiting step(s) of the hydrolysis of ATP; in terms of the current reaction scheme this step(s) follows the formation of the phosphoenzyme. The fact that both $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are positive indicates that the activation step is energetically unfavorable but entropically favorable. That is, the decrease in order compensates for the increase in enthalpy; mechanistically one could visualize this as an increase in randomness of the protein structure coupled with stretching of, or hindered rotation about, some bonds. The increase in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ below the transition temperature, indicating a change in the nature of the rate limiting step(s), suggests that the increased order of lipid, reflected in the reduced mobility of the spin label, imposes an increased order on the preactivation state, thereby producing an increased $\Delta S^\ddagger$, while also somehow energetically impeding the transition associated with activation. The difference between DPL-enzyme and SR can be similarly explained: the increased “order” in the lipid moiety of DPL-enzyme, resulting in an increase in both $\Delta H^\ddagger$ and $\Delta S^\ddagger$, produces not only greater loss on activation of rigidity or order (e.g. hydration) in the enzyme, but also increases the energy barrier for activation. The same reasoning would explain the larger $\Delta H^\ddagger$ and $\Delta S^\ddagger$ obtained with del-enzyme as compared to SR. In this case the reduction in lipid content results in an increased order of the lipid moiety, as discussed above, which in turn would affect the protein in the same way as lipid replacement by DPL, although the magnitude of the effects are much larger in the case of DPL replacement.

In the light of the above discussion, the large inhibition of phosphoenzyme decomposition in DPL-enzyme below its transition temperature might be attributed to stabilization of the phosphoenzyme form by the highly ordered array of the DPL molecules around the enzyme. Alkaline pH (25, 35), or high Ca$^{2+}$ concentrations (27) also exert a stabilizing effect on the phosphoenzyme form by the highly ordered array of the DPL molecules around the enzyme. Alkaline pH (25, 35), or high Ca$^{2+}$ concentrations (27) also exert a stabilizing effect on the phosphoenzyme decomposition in DPL-enzyme below its transition temperature, indicating a change in the nature of some bonds. The increase in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ below the transition temperature, indicating a conformational change that accompanies Ca$^{2+}$ translocation, in which the enzyme changes from PE(Ca)n to PE$^\ddagger$(Ca)n (Equation 7), resulting in net accumulation of PE(Ca)n, or at the level of the decomposition of the PE$^\ddagger$(Ca)n form (Equation 8), resulting in its net accumulation. In either case, it remains to be resolved whether the effect of high Ca$^{2+}$ or alkaline pH involves the same conformational stabilization that we postulate for the phospholipid effect; further experiments are needed to clarify this problem.

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