its entropy of formation may be interpreted to be nearly zero. It is tempting, therefore, to conclude that the basic idea behind the Page-Jencks model is applicable to elastase catalysis. A large part of the catalytic power of the enzyme may result from the rate-determining transacylation reactions occurring within a preexisting enzyme-substrate complex. The unusually favorable entropy of activation for these reactions could result in a greater than 10^4-fold rate acceleration over analogous bimolecular reactions.

Acknowledgment

I would like to thank Dr. E. R. Blout for his support and encouragement and Dr. W. P. Jencks for helpful criticism of this manuscript.

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Reversible Lipid Titrations of the Activity of Pure Adenosine Triphosphatase-Lipid Complexes†

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ABSTRACT: Using pure complexes of synthetic phospholipids with ATPase derived from sarcoplasmic reticulum, a lipid titration technique has been developed which enables a rapid preliminary screening of the effect of added lipids on ATPase activity. The data from the titration experiments are compared with the properties of some pure ATPase-lecithin complexes in which the activity of the ATPase is highly sensitive to the structure of the lipid chains. The activity of ATPase complexes with saturated lecithins is completely inhibited below temperatures which probably correspond to a phase transition of the lipids. Preliminary experiments indicate that the lipid titration technique can be readily adapted to a wide range of membranes.

We have prepared complexes of pure ATPase from sarcoplasmic reticulum with three synthetic lecithins to examine the effect of the thermal phase transition on the activity of the enzyme. Complexes of the ATPase with dioleoyllecithin (DOL, 18:1; 18:1), dipalmitoyllecithin (DPL, 16:0; 16:0), and dimyristoyllecithin (DML, 14:0; 14:0) provide evidence for a critical effect of the chain conformation on the activity of the ATPase.[1] The use of such complexes, containing essentially pure lipid and almost free of detergent, is obligatory for definitive studies of the effect of the lipid phase transition on ATPase activity, especially where the transition temperature of the lipid may be influenced by the presence of the protein.

However, the preparation of these complexes by the lipid substitution technique which we have described elsewhere is lengthy, involving successive lipid equilibrations in cholate, alternating with long centrifugation steps (Warren *et al.*,

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[1] Abbreviations used are: DOL, dioleoyllecithin; DPL, dipalmitoyllecithin; DML, dimyristoyllecithin; DSL, distearoyllecithin; DOPE, dioleoylphosphatidylethanolamine; SR, sarcoplasmic reticulum; glc, gas-liquid chromatography.

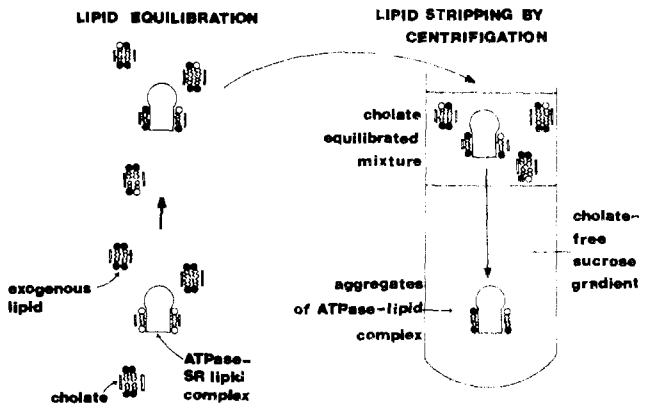

FIGURE 1: A diagrammatic representation of the equilibration and centrifugation steps of the lipid substitution procedure. The single shell of SR lipid surrounding the ATPase becomes completely equilibrated with the added exogenous lipid in the presence of cholate, by a mutual exchange process. In the centrifugation step, the excess lipid and most of the cholate remain in the supernatant layer, and the ATPase is recovered in a particulate form consisting of aggregates of the unit structure shown.

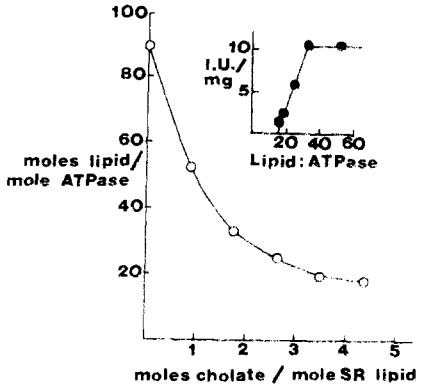

FIGURE 2: The effect of cholate concentration on the amount of SR lipid associated with ATPase, purified by the centrifugation technique at 4° (Warren *et al.*, 1974b,c); inset: ATPase activity at 37° as a function of lipid:enzyme molar ratio.

1974b,c). During the preparation of the complexes we found a simple and rapid titration procedure could be used to screen phospholipids quite accurately for interesting effects of phase transitions and chemical structure on the activity of the ATPase. This lipid titration technique depends on the equilibration in the presence of cholate, of lipids added to ATPase-lipid complexes. Simply by mixing a large excess of the lipids to be tested with a pure ATPase-lipid complex in cholate and following the subsequent change in ATPase activity to equilibrium, a clear indication of the effect of the test lipid on the ATPase can be obtained. We present evidence that the lipid titrations in cholate provide an effective screening procedure because cholate acts as an inert equilibrating agent for lipids and does not itself modify the action of the test lipids. More surprisingly, the effects of the thermal transition are preserved quite accurately in these titration complexes. We suggest that these phase transition effects are preserved because most of the cholate dissociates from the titration complexes when they are highly diluted into the ATPase assay medium. The ATPase-lipid complexes in the assay medium therefore contain a high proportion of the test lipid with low levels of bound detergent, and provide a reasonable approximation to the pure complexes obtained by complete lipid substitution and removal of detergent.

Preliminary experiments suggest that the titration technique is adaptable to a range of other membrane proteins. However, it is essential to validate titration results for each system by comparison with the properties of pure detergent-free complexes. We set out the conditions which probably have to be satisfied for the titrations to provide a reliable screening method.

Methods and Results

Preparation of Pure ATPase-Lipid Complexes. Since we are concerned mainly with the properties of pure ATPase-lipid complexes and the validity of lipid titrations in cholate as a screening technique, it is convenient to present the methodology with the results. The purification of ATPase-SR lipid complexes from the sarcoplasmic reticulum (SR) of rabbit muscle has been described previously (Warren *et al.*, 1974b). Complexes of ATPase with DML and DPL were prepared by minor adaptations of the lipid substitution technique previously used to prepare DOL-ATPase (Warren *et al.*, 1974b,c). The two basic lipid equilibration and centrifugation steps are illustrated in Figure 1. Briefly, the purified ATPase together with its associated SR lipid are suspended in cholate containing an excess of the pure exogenous lecithin. Under appropriate conditions of incubation, the two lipid pools equilibrate so that the lipid environment of the protein now reflects the composition of the entire lipid pool (Figure 1). The equilibrated mixture is then layered onto a sucrose gradient and subjected to a high centrifugal field. The ATPase with its associated lipid sediments through the sucrose gradient leaving behind excess lipid in the supernatant detergent layer. In addition, most of the associated detergent diffuses away from the sedimenting lipid–protein complexes. The ATPase-lipid complexes are collected from the sucrose gradient in a particulate form. Residual detergent can be removed by exhaustive dialysis in the presence of Amberlite XAD-2 resin (BDH). The process of lipid substitution can be readily followed by glc analysis of the methyl esters of the fatty acids derived from the lipids associated with the ATPase. After a second substitution step, more than 99% of the endogenous SR lipid can be replaced by DOL. Similar conditions can be used to prepare DML-ATPase, but higher temperatures (40°) are required to equilibrate DPL in cholate with endogenous SR lipid. The addition of 5 mM $MgATP^{2-}$ is also necessary to maintain the ATPase in an active conformation when equilibrating at higher temperatures. By these techniques, essentially pure, cholate-free complexes of DOL-, DML-, and DPL ATPase have been prepared. The lecithins were synthesized by the method of Robles and Van den Berg (1969); all other experimental techniques used have been described previously (Warren *et al.*, 1974b,c).

Several important features of the ATPase-lipid complexes and the action of cholate are illustrated in Figure 1 which are germane to the lipid titration technique described later. The ATPase-lipid complexes are shown as having a single shell of lipid bilayer surrounding the hydrophobic surface of the penetrant part of the protein and "solvated" with cholate molecules. The amount of lipid associated with the substituted ATPase after centrifugation depends on the molar ratio of cholate to lipid used in the equilibration (Figure 2). However, full ATPase activity is only maintained at a molar ratio of lipid to protein above about 30, below which there is an irreversible loss of activity to a negligible level at about 15 lipid molecules per ATPase (Figure 2, inset). For this reason in the preparation of ATPase-lipid

TABLE I

Lipid–Protein Complex	Molecules of Lipid/ Molecule of Protein[a]
Purified ATPase	33 (SR lipid)
DOL–ATPase	32
DML–ATPase	28
DPL–ATPase	26

[a] The amount of lipid associated with the ATPase is the minimum amount which will maintain maximal activity.

complexes we have adjusted the cholate to lipid ratio, to strip the enzyme of the maximum amount of lipid without loss of ATPase activity. About 30 lipids must be associated with each ATPase molecule, *irrespective of the structure of the lipid* (Table I) (Warren *et al.*, 1974a). Spin label experiments suggest that a shell of lipid bilayer surrounding the hydrophobic part of the ATPase is relatively immobilized compared with the rest of the lipid pool. It is therefore possible that the 30 lipid molecules interact directly with the ATPase to support full enzymic activity (P. A. Toon *et al.*, unpublished results). This model of the ATPase-lipid complex (Warren *et al.*, 1974a) is similar to that proposed previously for cytochrome oxidase–lipid complexes by Jost *et al.* (1973), also based on spin label studies.

The cholate is shown as causing complete mixing of the added lipid with the lipid associated with the ATPase. The evidence is that the levels of lipid substitution observed in the isolated complexes correspond to the levels calculated by assuming complete mixing of the lipid pools. The lipid mixing apparently occurs by an exchange process between the added lipid–cholate complexes and the ATPase–lipid–cholate complexes; this accounts for the constant lipid:protein composition of the isolated complexes irrespective of the amount of added lipid (Warren *et al.*, 1974b). The structure of the particulate ATPase–lipid complexes isolated from the sucrose gradient is assumed to be aggregates of the unit complex structure. In this form the vesicles do not accumulate calcium simply because they are too leaky (G. B. Warren *et al.*, unpublished results).

In the following titration experiments we use the term "mixing" to describe the action of cholate on the lipid pools, since we assume that the changes observed in ATPase activity reflect some mixing of the lipids. We do not imply that the mixing need be either complete or random as indicated in Figure 1, because this ignores the interesting possibility that the lipid pools around the protein may be segregated. This possibility is one reason why the titration results require confirmation by the preparation of completely pure complexes.

Reversible Lipid Titrations of ATPase Activity. The lipid titration experiments were performed by mixing a large excess of the lipid to be tested with a pure ATPase–lipid complex both dispersed in cholate (usually 0.5 mg of cholate/mg of total lipid). The subsequent change in ATPase activity of the mixture was followed to equilibrium, usually at 4°, using the coupled enzyme assay described previously (Warren *et al.*, 1974b). A feature of this assay is that when the ATPase–lipid–cholate dispersions are rapidly diluted 200–400-fold into the assay medium, most of the cholate dissociates from the lipid–protein complexes. If, for example, the ATPase–SR–lipid complex is treated with

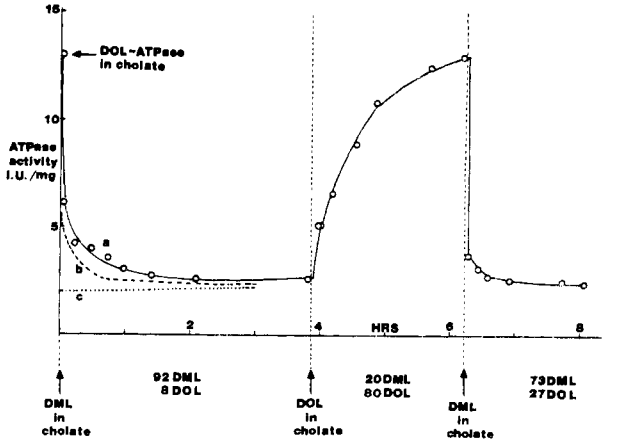

FIGURE 3: Titrations in cholate of DOL–ATPase activity with DML and DOL. DOL–ATPase (0.5 mg of protein) was incubated at 4° with 1.0 mg of DML and 0.5 mg of cholate in 0.20 ml of a buffer containing 50 mM potassium phosphate, 250 mM sucrose, and 1.0 M KCl, adjusted to pH 8.0. After 230 min, 4.0 mg of DOL and 2.0 mg of cholate in 0.80 of buffer were added, and after a further 140 min, a final addition of 10 mg of DML and 5 mg of cholate in 2.0 ml was made. ATPase activity was assayed at 37°. The effect of cholate concentration on the rate of equilibration of ATPase activity is also shown: DOL–ATPase was treated with DML as above at cholate concentrations (a) 2.5 mg/ml; (b) 5.0 mg/ml; and (c) 10 mg/ml, at a constant ratio of 0.5 mg of cholate/mg of DML.

DOL in cholate, diluted 200-fold into the assay medium, and then pressed through a 0.45-μ Millipore filter, 90% of the DOL–ATPase complex is retained but only 4% of the original cholate. Modifications of the above conditions to achieve mixing of DPL and other lipids are given in the figure legends.

An experiment is shown in Figure 3, in which DML–cholate has been added to DOL–ATPase also in cholate (DML–DOL, 9:1). The activity of the enzyme drops to about 15% of the activity of the DOL–ATPase at a rate which depends on the absolute cholate concentration at a constant ratio of 0.5 mg of cholate/mg of lipid (Figure 3). However, at each cholate concentration the ATPase activity at equilibrium is similar. If this lowering in activity represents mixing of the DML with the DOL around the protein, it should be reversible by adding a large excess of DOL in cholate. The restoration of ATPase activity to approximately its original level by DOL is also shown in Figure 3. It should be noted that the rate of reequilibration is slow compared with the initial action of DML and that further experiments have shown that reactivation by DOL is incomplete after long exposures of the ATPase to DML and cholate. Thus after 20 hr the enzyme cannot be reactivated by DOL, so that there appear to be slow secondary changes in enzyme structure in the presence of DML and cholate. Figure 3 shows that the enzyme activity can be lowered again by DML after DOL reactivation, confirming the reversibility of the titration. Qualitatively similar titration results are obtained with purified ATPase–SR–lipid complexes. The reduction in activity produced by DML can be back-titrated with SR lipid, or DOL, in the presence of cholate (Figure 4).

These experiments demonstrate that the action of DML is reversible and does not depend on the original lipid environment of the protein. There remains the possibility that the lowered activity results from a synergistic effect of cholate with DML. To resolve this, titration experiments have been performed in the absence of detergent, by mixing

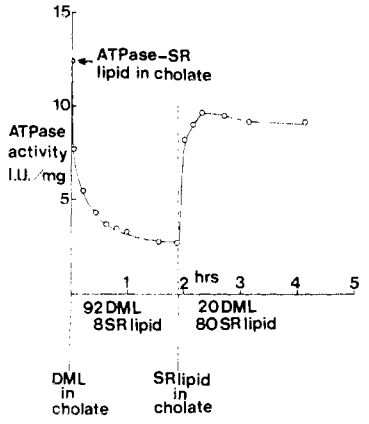

FIGURE 4: Titrations in cholate of purified ATPase–SR lipid complexes with DML and SR lipid. Purified ATPase (0.5 mg of protein) was incubated at 4° with 1.0 mg of DML and 0.5 mg of cholate in 0.20 ml of the standard buffer (see legend to Figure 3). After 110 min, 4.0 mg of SR lipid and 2.0 mg of cholate in 0.80 ml of buffer were added. ATPase activity was assayed at 37°.

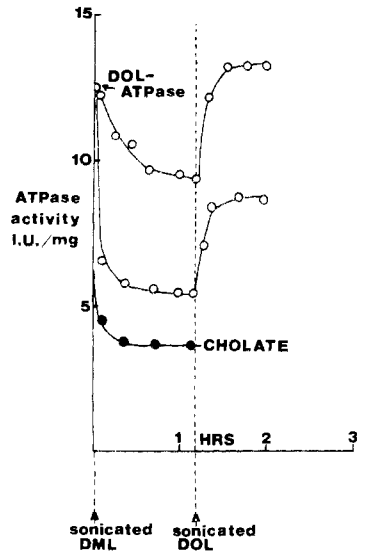

FIGURE 5: Fusion of sonicated lipid vesicles with pure DOL–ATPase in the absence of cholate. DOL–ATPase (0.5 mg) was incubated at 40° with 0.5 mg of highly sonicated DML in a total volume of 0.5 ml of the standard buffer to which substrates were added to stabilize the ATPase (5 mM ATP–5 mM $MgSO_4$). This corresponds to a molar ratio of lipids of about 85:15 DML–DPL. After 75 min, 4.0 mg of highly sonicated DOL in 0.5 ml of standard buffer with substrates was added. The results of two similar fusion experiments are shown. The differences in the degree of inhibition and reactivation reflect the incomplete mixing of the lipids by the fusion technique. Complete mixing of the DMl and DOL occurs on the addition of cholate which is shown for comparison.

highly sonicated lipid vesicles of DML with the ATPase–DOL complex. Fusion of the lipid vesicles with the ATPase–lipid complexes causes partial mixing of the lipid pools. Similar reversible activity changes are then observed to those obtained by the action of cholate on the lipid mixtures, although the magnitude of the changes varies significantly between experiments, presumably reflecting variation in the extent to which fusion has occurred (Figure 5). Since this is much more difficult to control precisely, the fusion technique is less convenient for screening lipids than the cholate titrations. Previous experiments have shown that lipid molecules in sonicated vesicles of spin-labeled lipid become mixed with endogenous SR lipid in native SR

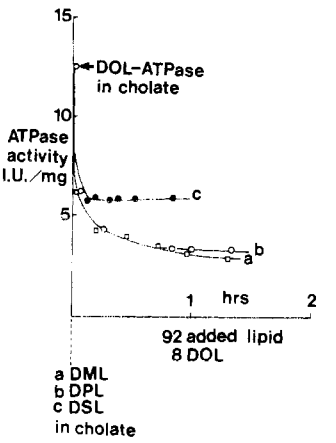

FIGURE 6: Titrations in cholate of DOL–ATPase with saturated lecithins: (a) DOL–ATPase treated with DML exactly as in Figure 3; (b) DOL–ATPase (0.5 mg of protein) was incubated at 40° with 1.0 mg of DPL and 0.5 mg of cholate in 0.20 ml of the standard buffer to which substrates were added to stabilize the enzyme (5 mM ATP–5 mM $MgSO_4$); (c) DOL–ATPase (0.5 mg of protein) was treated with 1.0 mg of DSL and 0.5 mg of cholate at 50° for 2 min and then allowed to equilibrate at 40° in 0.05 ml of the same buffer system with added substrates as in (b). All ATPase activities were assayed at 37°.

membranes when fusion occurs (Scandella *et al.*, 1972). The fusion experiments described here provide useful independent evidence for the effect of lipid structure on ATPase activity, and confirm that cholate acts essentially as an inert dispersing agent in these titrations.

Effect of Lipid Structure on ATPase Activity. Titration experiments in cholate can be used for a preliminary screening of the physical and chemical effects of phospholipid structure on the activity of the enzyme. For example, the titrations in Figure 6 suggest that when assayed at 37°, DPL lowers DOL–ATPase activity to about the same extent as DML, whereas distearoyllecithin (DSL) has a smaller effect. Since the effect of DSL could only be observed after brief exposure to relatively high temperatures (see legend to Figure 6), it was possible that the apparently smaller effect of DSL might result from inadequate mixing of the lipids. However, it was found that the action of DML could be partially reversed by DSL (Figure 7), consistent with an intrinsically smaller reduction in DOL–ATPase activity by DSL than DML. The time course of the reequilibration of ATPase activity during the back-titration is complex, with some overshoot in the activity in the initial stage, but the final level of activity at equilibrium is close to that obtained by direct titration of DOL–ATPase with DSL in cholate (*cf.* Figures 6 and 7). Dioleoylphosphatidylethanolamine (DOPE) will back-titrate DOL-ATPase treated with DML to approximately the same activity as DOL, suggesting that the two main lipid classes in SR (lecithins and phosphatidylethanolamines) have about the same effect on ATPase activity. This was confirmed more directly in preliminary experiments in which these two lipid classes were purified after chloroform–methanol extraction of SR lipid and found to back-titrate DOL–ATPase treated with DML to about the same activity. In similar experiments the unresolved mixture of negatively charged minor lipids (phosphatidylserines, phosphatidylinositols, and phosphatidic acids), which accounts for *ca.* 15% of the total SR lipid pool, also restored ATPase activity to about the same level as lecithin and phosphatidylethanolamines, so that there is no evidence at present for a lipid class in SR membranes which is able to lower the activity of DOL–ATPase.

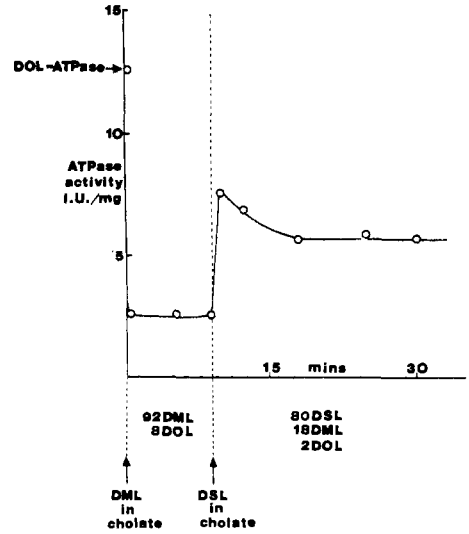

FIGURE 7: Back titration of ATPase activity by DSL. DOL-ATPase (0.5 mg of protein) was incubated at 40° with 1.0 mg of DML and 0.5 mg of cholate, in 0.05 ml of standard buffer, with substrate protection (see Figure 6b). After 10 min, 4.0 mg of DSL + 2.0 mg of cholate in 0.20 ml were added at 40°. ATPase activities were assayed at 37°.

These experiments therefore suggest that while saturated lipid chains maintain lower activity compared with oleoyl chains at all accessible temperatures, the structure of the polar head group appears to be much less important in determining activity.

Effect of Temperature on the Activity of ATPase-Lecithin Complexes. A preliminary indication of the effect of the phase transition in the saturated lecithins can be obtained from titration experiments. ATPase-SR-lipid complex was treated with a large excess of DPL in cholate and the ATPase activity of the equilibrated mixture was measured as a function of temperature (Figure 8a). Corresponding data for the pure, detergent-free DPL-ATPase included in Figure 8a show that the temperature profiles of activity are very similar for the complexes obtained by the titration and lipid-substitution techniques. Both complexes have appreciable activity down to about 28°, below which ATPase activity is completely inhibited. These results indicate that the temperature dependence of the enzymatic activity can be estimated quite accurately by the titration technique.

The quantitative comparison between the pure, detergent-free DML-ATPase and the complex prepared by lipid titration in cholate is less straightforward because of the intrinsic instability of the completely defined complex; in fact this instability is implied in the incomplete reactivation by DOL of ATPase treated with DML in cholate, which develops after long exposure. Thus, the activity of the completely defined complex is substantially lower at all assay temperatures than the titration complex (Figure 8b); however, both complexes are completely inhibited below 24°. This is close to the transition temperature of pure DML in the absence of protein of *ca.* 25° (Ladbrooke and Chapman, 1969) and is to be contrasted with the activity of DPL-ATPase complex well below the transition temperature of pure DPL at 42°. DOL has a phase transition at about −22° and DOL-ATPase is active at all accessible assay temperatures (Figure 8c).

It is clear that even in these fully defined systems in which the structure of the lipid component is known, the interpretation of thermal effects is complex. Nevertheless, the

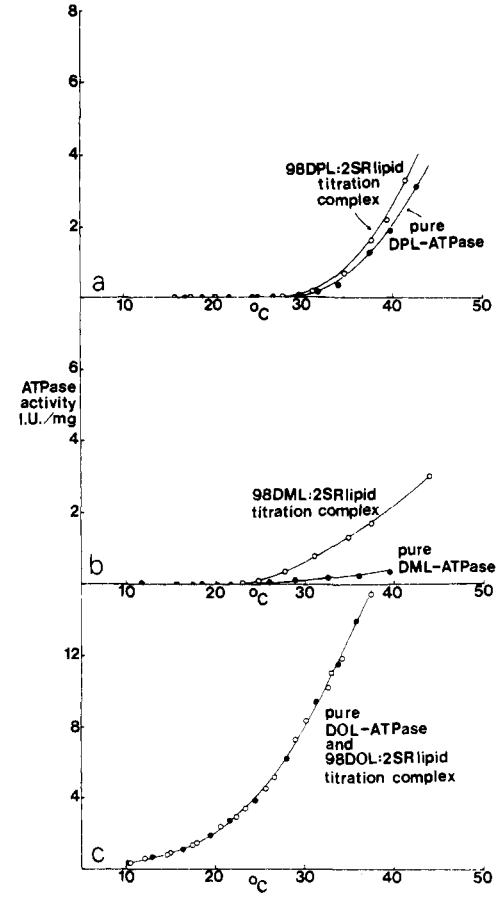

FIGURE 8: The temperature dependence of the activity of pure ATPase-lipid complexes compared with the complexes obtained by titration in cholate (see text).

ATPase activity appears to be sensitive to chain conformation as well as chain length and unsaturation, and activity is probably not maintained when the chains are in the fully extended all-trans configuration.

Discussion

Validity of the Titration Technique. The lipid substitution technique used to prepare pure ATPase-lipid complexes is effective because cholate and other detergents cause the mixed lipid pools to equilibrate rapidly and completely around the ATPase. The validity of the titration technique therefore hinges on whether the observed effects of lipids in the titration experiments apply also to cholate-free complexes, and whether the effect of a high proportion of a lipid is similar to that of complete substitution by the lipid. The available evidence strongly supports the conclusion that cholate acts as an inert equilibrating agent and does not itself significantly modulate the effects of different lipids on ATPase activity. Thus, the fusion experiments in which sonicated lipid dispersions produce similar qualitative changes in activity to the corresponding titrations in cholate clearly suggest that the activity changes are determined by the direct action of the lipids. The reversibility of the titrations precludes the possibility that inhibition is attributable to denaturation of the ATPase. The removal of cholate from the ATPase-lipid complexes by centrifugation and dialysis does not usually alter the enzymatic activity by more than ±10%, so that, for example, the cholate-free complexes of DOL- and DPL-ATPases have very similar activities to dispersions of the same complexes in cholate. It is

harder to establish whether the activity of complexes with a high proportion of a given lipid will in general be similar to the activity of the completely substituted enzyme. There is the possibility that as the last few lipid molecules around the ATPase are replaced, abrupt changes in activity may occur at a critical threshold of substitution. Alternatively, the activity of the enzyme may be considerably affected by segregation of the lipids when two or more lipid species are present, and this effect will be abolished when substitution is complete. A demonstration that either a threshold level of a particular lipid or that segregation of mixed lipid pools could determine the activity of the ATPase would be of interest, and it should be emphasized that there are at least these two potential sources of error in using the titration technique as a rapid screening method in systems where pure complexes have not been prepared. However, the data for the pure detergent-free DPL–ATPase and the corresponding titration complex demonstrate that in this instance there is no abrupt change in activity when substitution is complete.

There is more general potential objection to the use of detergents in this and other membrane systems. The purified ATPase isolated from SR by centrifugation out of a cholate dispersion is activated three- to fourfold compared with the corresponding steady-state ATPase activity of the enzyme in the original membrane. The activities of ATPase–lipid complexes defined by using this preparation refer to a protein structure which has a history of cholate treatment. We regard the profile of ATPase–lipid activities, which has been described, as relevant to the structure of the ATPase in the original membrane because the protein retains the substrate binding sites necessary for enzymatic activity and a functional capacity as a calcium pump in the reconstituted membrane vesicles (Warren *et al.*, 1974b). This strongly suggests that no essential features of the conformation have been lost and that the ATPase and ionophore activities remain coupled; the activation of the ATPase can be assumed to represent only a minor change in overall structure. We can also note that other mild treatments of the intact SR membrane also lead to some activation of the ATPase. Thus, exposure of SR to the ionophore X537A which renders the vesicles leaky to Ca^{2+} ions results in a threefold activation (Scarpa *et al.*, 1972). Fusion of intact SR with small amounts of sonicated DOL vesicles also produces an activation of 1.5- to 2-fold. Further evidence from other sources suggests that the initial turnover rate of the ATPase in SR immediately after the addition of ATP is more than tenfold faster than the steady-state level of activity (Yamada *et al.*, 1971).

More generally, it has been found that detergents often cause activation of membrane-bound enzymes or changes in the affinity of membrane receptors for ligands. In discussing the effect of detergents on the nicotinic (cholinergic) receptor, Changeux (1974) has commented that among several interpretations that can be proposed for the phenomenon, one is that solubilization by detergents releases a membrane constraint, created either by membrane lipids or proteins or both, to stabilize the molecule predominantly in an alternative conformation. Understanding this rather general phenomenon of detergent action on membrane proteins and the nature of the "membrane constraint" is likely to be important in describing the control of membrane function. One possibility we are exploring is that a nonrandom distribution of lipids in the plane of the native SR membrane or an asymmetric distribution of the lipids across the bilayer may serve to regulate the function of the ATPase. This lipid organization may be randomized irreversibly by the action of detergents and account for the activation of the enzyme.

Lipid Specificity of the ATPase. The titration experiments suggest that saturated lecithin chains containing up to 18 carbons substantially lower the activity of the ATPase compared with oleoyl chains, which confer the highest enzyme activity so far achieved. There appears to be some gradation in the effect in that stearoyl (18:0) chains cause a smaller reduction in activity than palmitoyl (16:0) chains at 37° There is some evidence that the DML–ATPase, which has 14:0 chains, is at the limit of structural stability. This is suggested by the intrinsic instability of the pure, detergent-free complex and the slow secondary changes in the structure of the DML–ATPase in cholate at 4°. All of the ATPase–lipid complexes are rapidly and completely inactivated by dilauroyllecithin (12:0 chains). It therefore appears that the hydrophobic part of the protein interacting with the lipids requires chains which are well tailored in length and unsaturated to maintain a functional conformation. The overriding importance of chain conformation is demonstrated by the effect of the phase transition. Although the bilayer becomes thicker below the transition, the enzyme is probably inhibited by the all-trans configuration. Neither DML- nor DPL- ATPase is phosphorylated in the inactive state and the temperature profile of the ATP-ADP exchange reaction catalyzed by these complexes coincides with the ATPase activity profile (G. B. Warren *et al.*, unpublished data). We can exclude the possibility that the enzyme is completely extruded from the bilayer below the transition, because it co-sediments with the lipid under these conditions. The requirement of the enzyme for a liquid-like chain environment does not preclude the possibility discussed elsewhere (Toon *et al.*, 1975) that the first shell of lipid bilayer surrounding the protein is distinguished by being relatively immobilized compared with the lipid unperturbed by interaction with the protein (Warren *et al.*, 1974a; Toon *et al.*, 1975). We cannot explain at present why the inhibition of the DML–ATPase coincides quite closely with the transition temperature of pure DML at 25°, while DPL enzyme has significant activity down to 29°, well below the transition at 42°. An obvious possibility to be examined is that the transition temperature of the lipid in the DPL–ATPase complex is substantially depressed by interaction with the protein. Definitive studies of the phase transition will require complexes of ATPase with lipid in which the residual cholate level can be shown to have no significant effect on the phase transition of the pure lipid.

In contrast to these experiments indicating an important role of the lipid chains in determining the activity of the ATPase, the structure of the polar head group appears to be less important. Thus, DOPE, DOL, and the unresolved fraction of minor lipids from SR will back-titrate the DML-treated enzyme to approximately the same activity. It should be noted that this conclusion refers explicitly to ATPase which has been exposed to cholate.

Recent lipid titration experiments in cholate on the Na^+K^+ ATPases of erythrocyte membranes and the sarcolemma membrane from skeletal muscle suggest that DML reversibly inhibits both enzymes below 25°. It would appear that the technique may be applicable to many membrane systems. We would like to reemphasize, however, that titration experiments should serve only as a preliminary screening technique, and for the reasons given previously, the re-

sults have to be compared with the properties of pure lipid–protein complexes.

Acknowledgments

We thank Mr. C. B. Turner for excellent technical assistance and the Science Research Council for partial support of this work.

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Phosphorylation of Endogenous Substrates by Erythrocyte Membrane Protein Kinases. I. A Monovalent Cation-Stimulated Reaction†

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ABSTRACT: Protein kinases in the isolated human erythrocyte membrane catalyze the transfer of ^{32}P from [γ-^{32}P]ATP to endogenous substrates, forming stable phosphopeptides. One of these reactions—phosphorylation of the major 215,000-dalton polypeptide—has several distinguishing features. The reaction is manifested at Mg^{2+} concentrations above 10^{-4} M and is relatively unresponsive to 20 μM cyclic AMP. It is stimulated by Ca^{2+} at 1 mM and by Na^+, K^+, Li^+, and NH_4^+ at 0.05 and 0.2 M. No turnover of this peptide-bound ^{32}P can be detected in the isolated membrane. The 215,000-dalton polypeptide is the only substrate utilized in this reaction. All other endogenous phosphorylations, particularly those strongly stimulated by cyclic AMP, are inhibited by Ca^{2+} and/or by monovalent cations. The specific substrate for the reaction is one of the major components of the elutable polypeptide mixture, "spectrin," and is released from the membrane at low ionic strength. However, the corresponding kinase is not eluted with its substrate: retention of the enzyme in membrane vesicles is demonstrable in reconstitution experiments. These properties are contrasted with those of the cyclic AMP-stimulated kinase activity, which is partially eluted at low ionic strength, while its principal substrates remain tightly bound to vesicles. The phosphorylation of an exogenous substrate, casein, by vesicles lacking "spectrin" is also stimulated by monovalent ions and thus resembles the endogenous reaction. Modes of ionic regulation and possible functional roles of the phosphorylation are discussed.

Erythrocyte membranes require ATP in the performance of a variety of functions. In the active transport of cations, ATP serves directly as a substrate and there is a fixed stoichiometry between ATP hydrolysis and the number of cations translocated. This type of function is well reflected in a specific component of Mg^{2+}-dependent ATP hydrolysis, the Na^+,K^+-stimulated ATPase (Skou, 1965). However, in ion transport, as well as other processes, ATP may participate through other mechanisms. In acting as an allosteric ligand or chelating agent, for example, ATP would not undergo hydrolysis. Likewise, its participation as a phosphate donor in transphosphorylation reactions of glycolysis and in regulatory phosphorylation of proteins need not be well reflected in "ATPase" activities as manifested by release of inorganic phosphate. Under conditions in which phosphorylation is not closely coupled to phosphate release from acceptor molecules, other methods must be used to study the activity and its regulation.

We reported previously that, in association with the Mg^{2+}-dependent, Na^+,K^+-stimulated ATPase reaction of the erythrocyte membrane, a lone membrane polypeptide of

† From the Diabetes Unit, Massachusetts General Hospital, Boston Massachusetts 02114 (J.A.), and the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545 (G.F.). *Received August 1, 1973.* Supported by a grant from the John A. Hartford Foundation, Inc., to the Diabetes Unit, Massachusetts General Hospital, and by U.S. Public Health Service Grant No. 12708 to the Worcester Foundation. J.A. was a recipient of a Research and Development Award from the American Diabetes Association and is currently an investigator of the Howard Hughes Medical Institute. G.F. was aided by a Cancer Research Scholar Award from the American Cancer Society, Massachusetts Division, Inc.