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Annular Lipids Determine the ATPase Activity of a Calcium Transport Protein Complexed with Dipalmitoyllecithin†

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ABSTRACT: Pure complexes of dipalmitoyllecithin (DPL, 16:0, 16:0) with Ca^{2+}, Mg^{2+} dependent ATPase from sarcoplasmic reticulum are unusual in retaining significant ATPase activity down to about 30 °C, well below the transition temperature of the pure lipid at 41 °C. A minimum of about 35 lipid molecules per ATPase is required to maintain maximal ATPase activity, but the complexes are progressively and irreversibly inactivated at lower lipid to protein ratios. Complexes containing more than the minimum lipid requirement show very similar temperature profiles of activity above 30 °C over a wide range of lipid to protein ratios, up to 1500:1. Spin-label studies indicate that, at lipid to protein ratios of less than about 30 lipids per ATPase, no DPL phase transition can be detected, but at all higher ratios, a phase transition occurs at about 41 °C. In all of these complexes there are breaks in the Arrhenius plots of ATPase activity at 27–32 °C and at 37.5–38.5 °C. Experiments with perturbing agents, such as cholesterol and benzyl alcohol which have well-defined effects on the DPL phase transition, indicate that these breaks in the Arrhenius plots of ATPase activity cannot be attributed to a depressed and broadened phase transition in the lipids near the protein molecules. These results are interpreted as evidence for a phospholipid annulus of at least 30 lipid molecules which interact directly with the ATPase and cannot undergo a phase transition at 41 °C. This structural interaction of the ATPase with the annular DPL molecules has a predominant effect in determining the form of the temperature–activity profiles. *However, the perturbation of the DPL phase transition does not extend significantly beyond the annulus* since a phase transition which starts at 41 °C can be detected as soon as extraannular lipid is present in the complexes. We suggest that it may be a general feature of membrane structure that penetrant membrane proteins interact with their immediate lipid environment so as to cause only a minimal perturbation of the lipid bilayer.

The sharp breaks, which occur in the temperature profiles of the activities of many transport proteins, have usually been attributed to lateral phase separations (Overath et al., 1970) or the formation of clusters in the lipid bilayer (Lee et al., 1974; Wunderlich et al., 1975). In bacterial membranes with high proportions of a single phospholipid, the breaks coincide quite closely with the phase separations which can be detected in the isolated lipids, implying that the membrane proteins sense a transition which is unaffected by the presence of the protein. Studies of the phase transitions in these membranes by calorimetry and x-ray diffraction have shown that most of the lipids are able to undergo a normal phase transition. Since the penetrant protein molecules in many membranes are only separated laterally by small numbers of lipid molecules, a membrane protein presumably cannot have an extensive effect on phase separations beyond the lipid in its immediate environment. In contrast, in model systems of pure lipid bilayers it is well-established that extraneous small molecules and various polypeptides and proteins (including cytochrome *c*, basic myelin (A1) protein, and polylysine) can either raise or lower the phase transition temperature of an extensive lipid bilayer, depending on the structure of the added component (Hui and Barton, 1973; Papahadjopoulos et al., 1975). This suggests the

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possibility that penetrant membrane proteins may be so designed that they cause a minimal, short-range perturbation of the bilayer, although it seems very unlikely that the lipids which interact directly with a protein could undergo a normal phase transition.

For most membrane proteins, the lateral range over which they exert a perturbation of the lipid phase transition is not accessible to biochemical analysis, because changes in the function of the protein coincide precisely with phase transitions in the unperturbed lipid bilayer. However, we have recently found that pure complexes of dipalmitoyllecithin (DPL[1]) with the Ca^{2+}, Mg^{2+} dependent ATPase from sarcoplasmic reticulum are anomalous in retaining ATPase activity well below the phase transition at 41 °C (Warren et al., 1974a). We emphasize that the properties of the DPL–ATPase complex are unusual; in complexes with dimyristoyllecithin, inactivation of the ATPase coincides with the transition temperature of the lipid. This corresponds to the normal pattern of behavior of many other membrane proteins exemplified by the reactivation of Na^+, K^+ ATPase by synthetic phosphatidylglycerols (Kimelberg and Papahadjopoulos, 1974). Here we have exploited the unusual properties of the DPL–ATPase complexes to define the lateral range of bilayer perturbation, by varying the proportions of lipid and ATPase in the complexes and following the effect on ATPase activity and the DPL phase transition. These experiments point to a predominant effect on ATPase activity of about 30 lipid molecules, termed the annulus (Warren et al., 1975a), which interact directly with the ATPase and are unable to undergo a phase transition.

Materials and Methods

Sarcoplasmic reticulum (SR) was prepared from rabbit skeletal muscle as described previously (Warren et al., 1974b). The ATPase was purified with the 30 molecules of SR lipid per molecule of ATPase necessary for full ATPase activity, by treating SR with cholate and centrifuging into a sucrose gradient (Warren et al., 1974a,c). The 30 Sr lipid molecules were replaced by dipalmitoyllecithin (DPL) by suspending 200 mg of the purified protein with its associated lipid in a mixture of 400 mg of DPL and 200 mg of cholate in a total volume of 6.0 ml of 250 mM sucrose, 1 M KCl, 5 mM Mg ATP^{2-}, 50 mM potassium phosphate, pH 8.0 (sucrose buffer). After incubation at 40 °C for 30 min, 1.0-ml aliquots were layered on 2.5 ml of 30% sucrose, 0.2 ml of 50% sucrose, 0.2 ml of 80% sucrose, each in 1 M KCl, 5 mM Mg ATP^{2-}, 50 mM potassium phosphate buffer, pH 8.0. The tubes were centrifuged at 300 000*g* for 6 h at 35–36 °C and the pellets were pooled and washed twice in sucrose buffer to remove residual cholate. This procedure replaces about 90% of the endogenous SR phospholipid by DPL. To prepare complexes of the protein with different proportions of DPL, 100 mg of the partially substituted ATPase was treated with 300 mg of DPL in a total volume of 5 ml of 250 mM sucrose buffer. The mixture was sonicated by suspending a glass vial in a Megason ultrasonic cleaning bath (Shuco International Ltd.) for three bursts of sonication for 5 min each at 40 °C. Aliquots (1.0 ml) were then treated with 2, 10, 15, 20, or 25 mg of cholate (100 mg/ml) in sucrose buffer, incubated at 40 °C for 30 min and centrifuged as described previously. The isolated DPL–ATPase complexes contained 67, 43, 29, 25, and 17 mol of DPL/mol of ATPase protein, respectively. Residual endogenous lipid accounted for less than 2% of total lipid in any of these DPL–ATPase complexes. Each of these complexes was washed extensively in sucrose buffer until the level of residual cholate had fallen to less than 1 molecule of cholate per molecule of protein, and the samples were then frozen in liquid nitrogen and stored at −20 °C.

The complex containing 1500 molecules of DPL per molecule of ATPase was prepared by incubating 20 mg of the partially substituted DPL–ATPase with 75 mg of freshly sonicated DPL in 1.75 ml of sucrose buffer at 40 °C. Cholate (0.25 ml of 100 mg/ml in sucrose buffer) was then added and after incubation at 40 °C for 10 min the mixture was diluted slowly to 20 ml with ice-cold buffer containing 1 M KCl, 5 mM $MgATP^{2-}$, 50 mM potassium phosphate, pH 8.0, and centrifuged at 95 000*g* for 20 min at 5 °C. The pellet (about 10% of the total ATPase activity) was discarded and the supernatant was dialyzed against sucrose buffer for 16 h at 5 °C. It was then concentrated on an Amicon ultrafilter fitted with a PM-10 membrane, frozen in liquid nitrogen, and stored at −20 °C. A complex containing 900 molecules of DPL per ATPase was prepared by the same technique.

A complex of the ATPase protein with DPL and cholesterol (in a ratio of 2:1) was prepared in a manner similar to that described above for the 1500:1 DPL:ATPase complex except that 20 mg of the partially substituted DPL–ATPase was incubated with 40 mg of DPL and 10 mg of cholesterol which had been dried from a mixture in chloroform before adding sucrose buffer and sonicating to clarity at 45–50 °C.

The ATPase activity of the protein was assayed using a double-enzyme-coupled assay (Warren et al., 1974b). The protein–lipid complex (50–100 μg) was preincubated in the assay medium at the assay temperature for 10 min before the reaction was started by the addition of Ca^{2+}. This procedure eliminated an acceleration of the ATPase activity which was observed when the protein itself was used to start the reaction. ATPase activities in the presence of 50 mM benzyl alcohol were carried out by dissolving benzyl alcohol in the assay medium before the addition of the ATPase.

ESR spectra were recorded on a Varian E3 spectrometer and the temperature was monitored continuously during the experiment by a thermistor inserted immediately above the cavity. Control experiments showed that the temperature gradient in the cavity was less than 0.5 °C under the operating conditions used. The samples were inserted in 1.0-mm i.d. glass capillaries and the power level was adjusted to ensure that local heating did not affect the observed transition temperature of DPL. The membranes were labeled with 5-doxylstearic acid, 16-doxylstearic acid, or 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) at a molar ratio of membrane phospholipid to label of 100:1.

The fatty acid composition of phospholipids was analyzed by GLC of the transesterified fatty acids after chloroform–methanol (2:1) extraction (Warren et al., 1974b). DPL was prepared by the method of Robles and Van den Berg (1969) and protein was estimated by microbiuret determination (Goa, 1953).

Results

(*i*) *ATPase Activity and the DPL:ATPase Ratio.* In order to determine the effect of lipid to protein ratio on ATPase activity, complexes of DPL–ATPase were prepared with increasing proportions of DPL. At 37 °C the activity of the complexes was virtually constant above a molar ratio of about 35 lipids per ATPase, but decreased rapidly at lower propor-

[1] Abbreviations used: SR, sarcoplasmic reticulum; DPL, dipalmitoyllecithin; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; doxyl refers to the 4′,4′-dimethyloxazolidine-*N*-oxyl ring; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance.

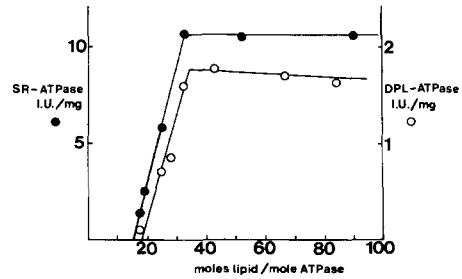

FIGURE 1: Activities of DPL-ATPase and SR-ATPase complexes as a function of lipid:protein molar ratio at 37 °C. It should be noted that, although the precise form of the profile for DPL-ATPase varies significantly with temperature, the intercept remains at about 35 DPL molecules per ATPase.

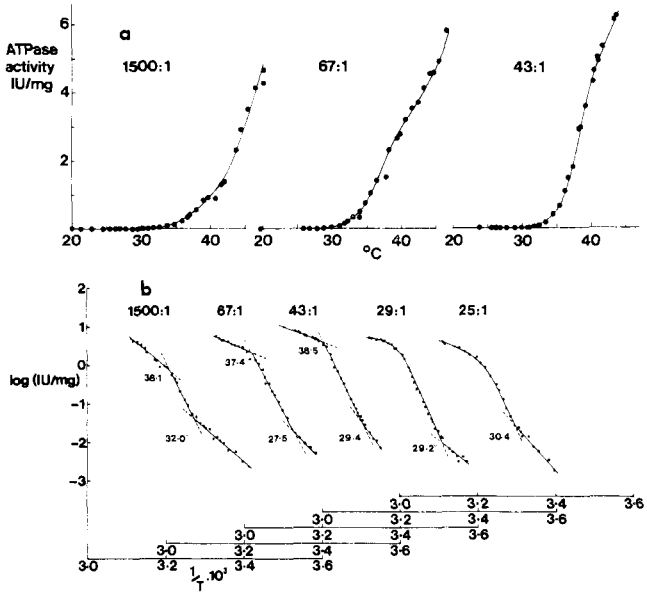

FIGURE 2: Activities of DPL-ATPase complexes as a function of temperature. In Figure 2b the lower temperatures at which breaks occur in the Arrhenius plots are not defined to better than ±1.5 °C because of the the very low values of the ATPase activities.

tions of lipid (Figure 1). This dependence of ATPase activity on the proportion of lipid in the complex is similar to that described previously for the ATPase complexed with endogenous SR lipid (SR-ATPase), although the maximal activity of the SR-ATPase complexes is much higher than for the DPL-ATPase complexes.

(ii) Temperature-Activity Profiles and the DPL:ATPase Ratio. The temperature profiles of the ATPase activity of three complexes with different DPL:ATPase ratios are compared in Figure 2a. Although the detailed form of the profiles above 30 °C varies with the proportion of DPL, all three complexes become very inactive below 30-32 °C (less than 1% of the activity at 45 °C). A similar inactivation of overstripped complexes containing less than 30 DPL molecules per ATPase also occurs at 30-32 °C. These results indicate that the temperature at which loss of ATPase activity occurs does not depend significantly on the DPL:ATPase ratio.

The apparent effect of the lipid phase transition in DPL at high DPL:ATPase ratios can be detected in detailed Arrhenius plots of the ATPase activity (Figure 2b). At high lipid to protein ratios there are two inflections in the Arrhenius plots at approximately 27-32 and 37.5-38.5 °C. As the proportion of DPL decreases the upper break remains in the same temperature range, but eventually broadens into a continuous

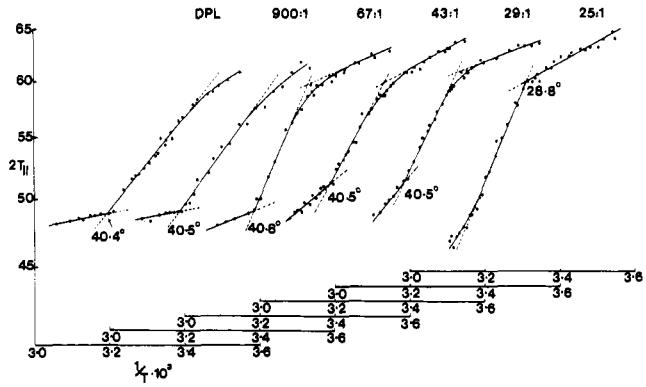

FIGURE 3: $2T_{\|}$ (gauss) as a function of $1/T$ for DPL-ATPase complexes (26 mg of protein/ml), spin labeled with 5-doxylstearic acid (1 mol % of total lipid).

curve below a ratio of 30 DPL:ATPase. At the same time, the lower break also remains at about 27-30 °C, but becomes more pronounced. The complexes with very low DPL:ATPase ratios are therefore characterized by a well-defined break at about 27 to 30 °C and a continuous decline in activation energy at higher temperatures. No break at the transition temperature of DPL (41 °C) was detected in any of the complexes. The similarity of the Arrhenius plots in Figure 2b also emphasizes the relatively small effect on the temperature profile of the proportion of DPL in complexes with a complete annulus.

(iii) Spin-Label Assays of the DPL Phase Transition in DPL-ATPase Complexes. A clear indication of the effect of the ATPase on the DPL bilayer is obtained by using the 5-doxylstearic acid spin label. The Arrhenius plot of $2T_{\|}$ provides an arbitrary measure of the immobilization of the spin label, which shows a well-defined break at 40.4 °C for pure DPL coinciding with the phase transition (Figure 3). As the proportion of ATPase in the complexes is increased, the transition remains close to 41 °C but becomes less pronounced, and is not detectable at DPL:ATPase ratios below 25:1. Simultaneously the curvature in the Arrhenius plots at lower temperatures increases with the proportion of ATPase in the complexes, and the temperature at which there is a sharp decrease in $2T_{\|}$ is defined arbitrarily from the tangents to the curves as shown in Figure 3. The intercept occurs at 27 to 29 °C for all the complexes where the effect of the protein on the curve can be clearly detected (DPL:ATPase 67:1). At very high DPL:ATPase ratios, the curves were indistinguishable from the pure DPL curve.

Similar control experiments were performed with SR-ATPase, SR lipid, and intact SR vesicles to establish whether the spin-label parameters described are characteristic of the phospholipid present in the complexes. The plots in Figure 4 show that $2T_{\|}$ increases in the order SR lipid < SR < SR-ATPase, consistent with an increased immobilization of the label due to the ATPase, since there are about 100 lipid molecules per ATPase in native SR compared with a ratio of about 30:1 in SR-ATPase. The differences in $2T_{\|}$ between the samples were larger at lower temperatures, which may be attributable to a change in the distribution of the spin label between the protein and lipid environment in favor of the lipid bilayer as the temperature increases (see also 16-doxylstearic acid data below). The plot for SR-ATPase was approximately linear from 5 to 50 °C, unlike any of the DPL-ATPase complexes. Both the SR and SR lipid plots were curved with poorly defined breaks between 20 and 30 °C, clearly distinguishable from the DPL transition at 41 °C (Figure 4).

A further assay of the DPL transition in the DPL-ATPase

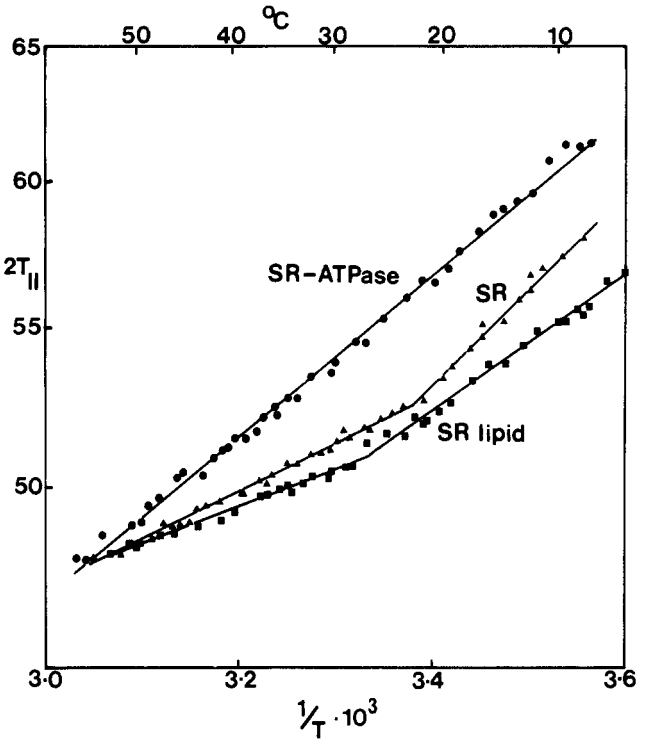

FIGURE 4: $2T_{\parallel}$ (gauss) as a function of $1/T$ for SR-ATPase (30 mg of protein/ml), nature SR (26 mg of protein/ml), and SR-lipid (100 mg/ml), spin labeled with 5-doxylstearic acid (1 mol % of total lipid).

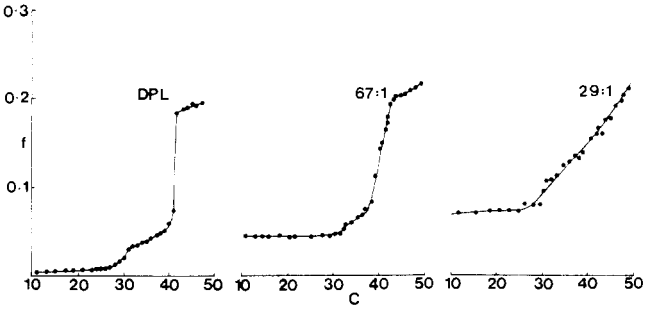

FIGURE 5: Fraction of Tempo (1 mol % of total lipid) bound to DPL and DPL–ATPase complexes (26 mg of protein/ml) as a function of temperature. The DPL concentrations in the samples were: 5 mg/ml (29:1); 11 mg/ml (67:1); and 10 mg/ml (DPL).

complexes was performed with the spin label Tempo which defines the transition as a large increase in the partition coefficient of the label into the bilayer, centered at 41 °C (Figure 5). There is also a pretransition, which starts at about 30 °C, and other spin-label studies indicate an increase in mobility in the DPL bilayer between 30 and 40 °C (Hubbell and McConnell, 1971; Oldfield et al., 1972). At very high DPL:ATPase ratios, the Tempo binding curve is unaltered, but at 67:1 DPL:ATPase there is a small increase in binding which starts at 32 °C and a steep increase between 37 and 43 °C, above which the binding is almost constant. At 29:1 DPL:ATPase there is a continuous slow increase in binding between 30 and 48 °C with no indication of a well-defined transition. Binding curves for overstripped complexes were unreliable because of the rapid reduction of Tempo by the ATPase and the low concentration of DPL in these complexes.

We conclude from the spin-label data that the ATPase eliminates the normal phase transition in DPL at molar ratios below about 30 DPL molecules per ATPase. The spectra observed with both 5-doxylstearic acid and Tempo may indicate a significant increase in the fluidity of the DPL annulus starting at about 28–30 °C, coinciding with the development of significant ATPase activity, although the spin-label evidence is not conclusive. Both labels indicate that an increasing proportion of the DPL molecules undergo a phase transition at close to the normal transition temperature as the proportion of DPL in the complexes is increased.

(*iv*) *Distinguishable Lipid and Protein Environments in DPL:ATPase Complexes.* The spectra observed from the 5-doxylstearic acid label approximate to a single environment for the spin labels over the temperature range from 5 to 50 °C. This is presumably because the immobilization of the label is similar both in the environment of the protein and in the DPL bilayer not interacting directly with the protein, and a single $2T_{\parallel}$ value can be measured. However, the spectra from the 16-doxylstearic acid label are sufficiently different in the two environments that partial resolution is observed (Figure 6a). The more immobilized component of the spectrum in DPL–ATPase complexes is not present in spectra from the label in DPL alone and can be attributed to spin label bound onto or in the environment of the protein. The more fluid component of the spectrum is very similar to that observed in DPL. The composite spectrum is therefore derived from spin labels in two environments exchanging at a rate which is slow compared with their separation in Hz ($\simeq 10^6\ s^{-1}$). These spectra and their assignment to lipid and protein environments in the complexes are similar to those reported previously by Jost et al. (1973) for pure cytochrome oxidase–lipid complexes. The clear resolution of the two environments for the 16-doxylstearic acid label, compared with the unresolved spectra for the 5-doxyl analogue, suggests that the fluidity gradient in lipid chains in the immediate vicinity of the protein may be substantially less than the gradient observed in the lipid bilayer.

The proportion of the 16-doxyl acid label in the protein environment increases with the proportion of protein in the complexes as expected (Figure 6a), but the *distribution* of label between the two environments apparently changes substantially in favor of the lipid in all of the complexes as the temperature is increased (Figure 6b). This may reflect the enhanced partition of the label into the DPL bilayer when it provides a sufficiently fluid environment. We note that, if the differential partition of the 16-doxylstearic acid label between the lipid and protein environments is sensitive to temperature in this way, then the relative proportions of the label in the two environments cannot be used to quantitate the binding capacity of the protein for phospholipid chains. There is a further complication that the spin labeled fatty acid may bind to the ATPase at sites not normally accessible to the chains of the lipid molecules in the phospholipid bilayer. For these reasons, quantitative estimates of the proportion of phospholipid molecules interacting with the ATPase have to be made using spin-labeled phospholipids, rather than the fatty acid labels. If the 5-doxylstearic acid label also partitions preferentially into the DPL bilayer with increasing temperature, this would account for the facility with which the normal DPL transition is detected in extraannular lipid, even at low DPL:ATPase ratios.

Effect of Cholesterol and Benzyl Alcohol on the Complexes. One interpretation of the two breaks in the Arrhenius plots of DPL–ATPase activity is that they represent the two ends of a broadened and depressed DPL phase transition. If this is correct, then the Arrhenius plots of activity should respond to the presence of extraneous molecules in a manner which is consistent with their known effects on the DPL phase transi-

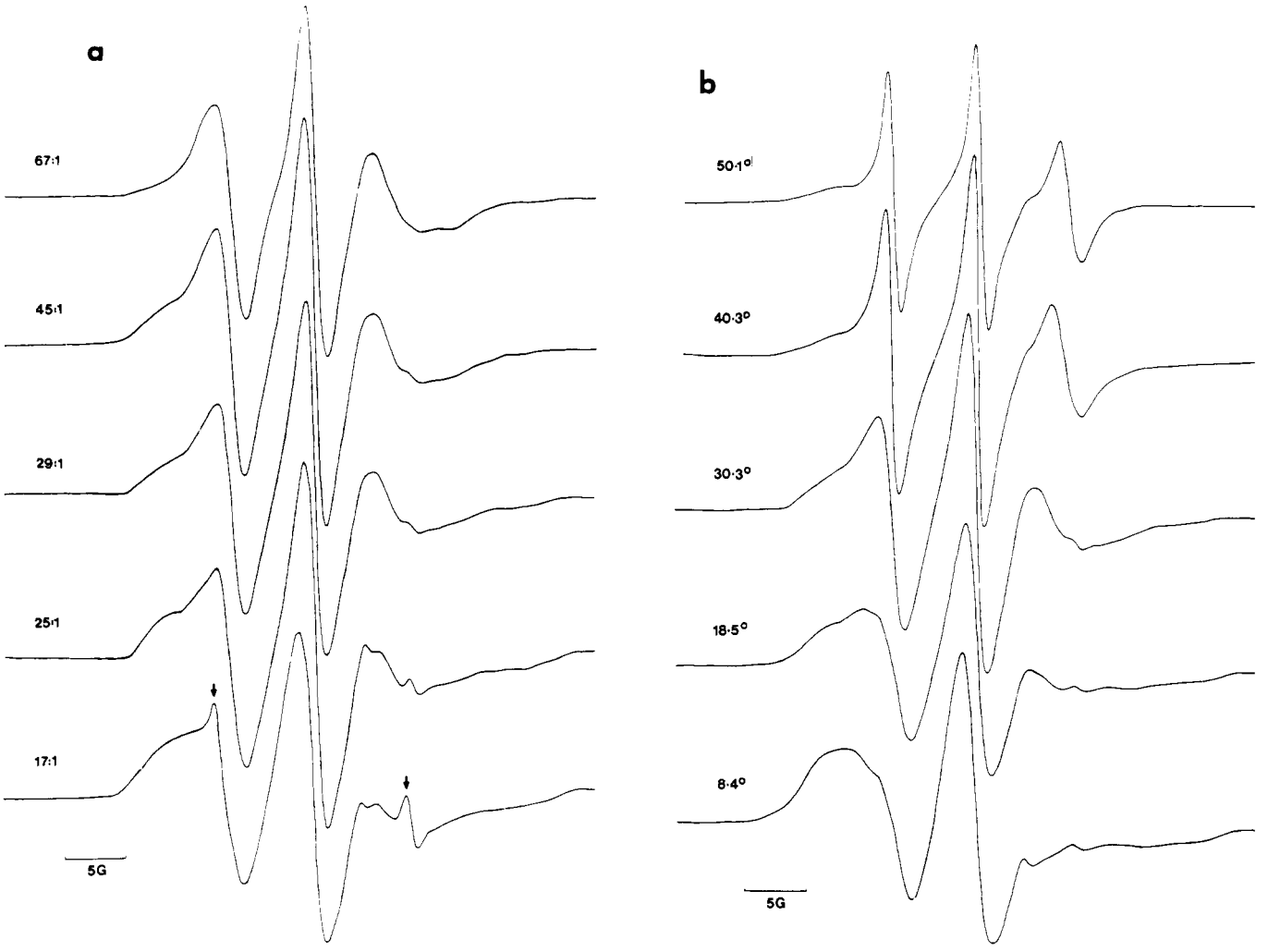

FIGURE 6: Spectra of 16-doxylstearic acid (1 mol % of total lipid) in DPL–ATPase complexes (26 mg of protein/ml) (a) as a function of lipid:protein molar ratio at 30 °C; (b) as a function of temperature for the complex containing 29:1 DPL:ATPase. The arrows indicate the position of unbound spin label which can be detected at low lipid:protein ratios.

tion. We selected cholesterol which broadens the phase transition of DPL beyond detection at a molar ratio greater than 2:1 DPL:cholesterol, and benzyl alcohol which depresses the transition temperature by about 6 °C at 80 mM (Colley and Metcalfe, 1972). In Figure 7b it can be seen that cholesterol does not significantly affect the upper break in the Arrhenius plot whereas the lower break is no longer observed. Similarly, in the presence of 50 mM benzyl alcohol, the upper break is retained, although slightly depressed to 36 °C, and the lower break is abolished. At the same time the alcohol activates the complexes by up to tenfold below the transition temperature, and by a decreasing amount at higher temperatures. It is important to note that this activation is fully reversible and presumably reflects a perturbation of the lipid–protein interaction. In contrast to the biochemical data, the 5-doxylstearic acid spin label provides evidence consistent with the expected effects of cholesterol and benzyl alcohol on the DPL phase transition in DPL–ATPase (130:1) complexes (Figure 7b). No phase transition was observed in the DPL/cholesterol (2:1)–ATPase complex, and in the presence of 50 mM benzyl alcohol there was a break in $2T_{\parallel}$ for DPL–ATPase centered at 34 °C. Similar effects of alcohol and cholesterol were observed in pure DPL bilayers (not shown). These spin-label experiments, therefore, confirm that, in the complexes of DPL with ATPase, there are extensive areas of DPL bilayer undergoing normal phase behavior.

Discussion

The main features of the biochemical and spin-label experiments provide a clear indication of the short-range perturbation of the DPL bilayer caused by an ATPase molecule, and a predominant effect of the annular lipids in determining the form of the temperature–activity profile of the enzyme in DPL–ATPase complexes.

The Major Perturbation of the Phase Transition in the Bilayer is Confined to the Annulus. The temperature profile of ATPase activity shows no systematic change with increasing proportions of DPL in the complexes, and the breaks in the Arrhenius plots of activity do not coincide with the normal phase transition of DPL. However, a DPL phase transition can be detected in all complexes which contain more than about 30 DPL molecules per ATPase, and the ATPase requires at least this number of lipid molecules for maximal activity. Thus each ATPase molecule prevents a normal phase transition occurring in about 30 DPL molecules, and experiments with spin-labeled phospholipids indicate that approximately the same number of lecithin molecules interact directly with the ATPase (Montecucco et al., 1976, in preparation). We therefore suggest that the first shell of phospholipid molecules interacting directly with the ATPase consists of an annulus of about 30 lipid molecules, and that the lipid molecules immediately outside the annulus are able to undergo a normal DPL

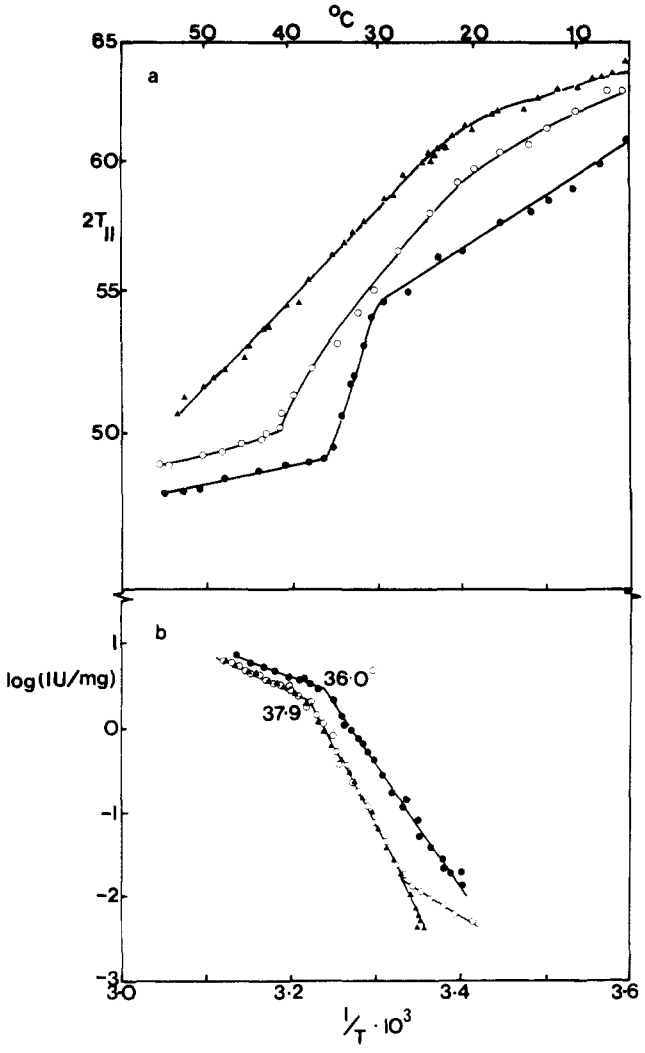

FIGURE 7: (a) $2T_{\parallel}$ (gauss) as a function of $1/T$ for DPL:cholesterol (2:1)-ATPase (▲) and DPL-ATPase ± 50 mM benzyl alcohol (●, ○), spin labeled with 5-doxylstearic acid (1 mol % of total lipid). The protein concentration in the samples was 25 mg/ml and the DPL:ATPase ratio was 130:1. (b) Arrhenius plots of the ATPase activities of the same three complexes.

phase transition. Recent experiments using carbon-13 enriched DPL have confirmed the results using spin labels. In a complex containing 140 DPL molecules per ATPase, at least 70% of the lipid went through a phase transition between 41 and 35 °C as evidenced by the change in area of the ^{13}C NMR spectra.

These conclusions are consistent with qualitative evidence from freeze-fracture pictures of the DPL-ATPase complexes, which show patches enriched in protein particles separated by striated areas characteristic of the DPL bilayer below the phase transition. At very high lipid to protein ratios, the ATPase particles frequently collect in a linear array aligned with a DPL striation, similar to those observed for dimyristoyllecithin (DML)-ATPase by Kleeman et al. (1974). It is possible that some areas enriched in ATPase particles contain only annular DPL which does not crystallize, although we have not been able to assess this critically from freeze-fracture pictures. However, it is clear that, in forming a protein-enriched phase, the lateral extent of bilayer perturbation does not increase significantly as the proportion of extraannular DPL is increased.

A Short-Range Lateral Perturbation of the Bilayer by Penetrant Proteins May Be a General Structural Feature of the Membrane. The short-range perturbation of the DPL bilayer by the ATPase suggests a more general prediction that those penetrant membrane proteins which have functions that respond directly to phase separations in the lipid bilayer will also cause short-range perturbations restricted mainly to their annular lipids. We suggest that the interaction of the annulus with the protein is designed to provide the protein with a lipid shell which causes a minimal perturbation of the subsequent lipid shells. In this way the membrane protein can respond to phase separations in a bilayer which is apparently unperturbed by the presence of the protein, and it is not necessary to assume that the lipid interacting directly with the protein takes part in a normal phase separation.

Spin-label data from both spin-labeled fatty acids and phospholipids suggest that the rate of exchange between annular lipids and the extraannular lipid shell is slow, at least compared with the rate of lateral diffusion in the lipid bilayer. Under special circumstances, where the ATPase is present in a lipid pool in which one phospholipid species is excluded from the annulus, the half-time for exchange is at least of the order of minutes (Warren et al., 1976, in preparation). We therefore regard the dynamic structure of the complex for rotational and lateral diffusion as the ATPase together with its associated annulus. This implies a special boundary between the annular lipids and the next lipid shell, which we have suggested previously is a major site of nonspecific leakage in SR vesicles (Warren et al., 1975b), and is responsible for the increased permeability of the membrane to ions compared with the permeability of a bilayer composed of the same lipids but in the absence of the ATPase (Martonosi et al., 1974). A critical test of this hypothesis using resealed DPL-ATPase complexes will be described elsewhere.

Annular Interactions of DPL with ATPase. A conventional interpretation of the breaks in the Arrhenius plots of DPL-ATPase activity would attribute them to the two ends of a DPL phase transition broadened and depressed by the presence of the ATPase. If this is correct, then the effects of cholesterol and benzyl alcohol on the Arrhenius plot should be consistent with their effects on the DPL phase transition. However, both cholesterol and benzyl alcohol eliminate the lower break at 27-32 °C whereas the upper break is retained. Since a DPL transition cannot be detected in the presence of cholesterol at a molar ratio of 2:1 DPL:cholesterol and benzyl alcohol merely depresses the transition, their effects on DPL-ATPase activity cannot be related simply to a modified DPL phase transition in or near the annulus. The observations are rationalized more easily by assuming that the Arrhenius plots represent some cooperative structural property of the interaction of the annular DPL molecules with the ATPase. A likely structural parameter is the *rigidity* of the annular lipids, which may directly affect the conformational flexibility of the ATPase. The suggestion is consistent with the substantial activation caused by benzyl alcohol over the subtransition temperature range, even though the alcohol itself is unable to support ATPase activity when it displaces annular phospholipid. The activation is presumably caused by the fluidizing effect of the alcohol on the rigid constriction imposed by the DPL annulus on the ATPase. We assume that, for ATPase complexes with lipids with high phase transition temperatures, such as DPL, there is sufficient residual fluidity in the annular lipids to support some ATPase activity, even when the extraannular lipids crystallize. This would account for the very high activation energy of the complexes below the transition and the diminishing extent of alcohol activation above the transition. For complexes of the ATPase with lecithins with lower transition temperatures such

as dimyristoyllecthin, functional inactivation occurs at the transition temperature of the pure lipid (Warren et al., 1974a). In these complexes, which exemplify the normal type of behavior of many proteins, we assume that crystallization of the extraannular lipid at the transition temperature imposes sufficient rigidity on the annular lipids to switch off activity.

Finally we wish to emphasize that we are not suggesting that the structural interaction of the annular lipid with the ATPase is independent of the properties of the lipid. This would imply a uniform temperature–activity profile for all complexes which is incompatible with previous data for the ATPase and many other membrane-bound enzymes. The specific conclusion which the anomalous behavior of the DPL–ATPase allows us to make is that the lipid phase transition is not greatly perturbed beyond the immediate lipid neighbors of the protein, and we suggest that the same conclusion will apply also to membrane proteins which show a normal dependence of function on lipid phase transitions.

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Temperature-Dependent ^{13}C Nuclear Magnetic Resonance Studies of Human Serum Low Density Lipoproteins†

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ABSTRACT: The natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum of human serum low density lipoproteins (LDL) shows significant temperature-dependent changes. These temperature-dependent spectra have been used to monitor changes in the organization of cholesterol esters within the LDL particle. Comparison with ^{13}C NMR spectra of both cholesterol linoleate and an aqueous codispersion of cholesterol linoleate and egg phosphatidylcholine suggests that at low temperatures (10 °C), the cholesterol esters in LDL are organized in a smectic-like, liquid-crystalline arrangement. At temperatures above the order–disorder transition exhibited by the cholesterol esters of LDL, the cholesterol esters appear to be partially melted but still are motionally restricted compared with liquid cholesterol esters.

Electron microscopy and small angle x-ray scattering indicate that human serum low density lioprotein (LDL[1]) is a spherical particle about 220 Å in diameter (Forte et al., 1968; Mateu et al., 1972). Recent differential scanning calorimetry and x-ray scattering studies show that the core of LDL is occupied mainly by cholesterol esters which undergo a temperature-dependent structural reorganization in the range of body temperature (Deckelbaum et al., 1975). Furthermore, the x-ray scattering shows that below this thermal transition, the

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[1] Abbreviations used: LDL, low density lipoproteins; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane; EDTA, (ethylenedinitrilo)tetraacetic acid.