Biochimica et Biophysica Acta, 598 (1980) 502—516

BBA 78746

PROTEIN-LIPID INTERACTION

BIOPHYSICAL STUDIES OF (Ca^{2+} + Mg^{2+})-ATPase RECONSTITUTED SYSTEMS

JUAN C. GOMEZ-FERNANDEZ *, FELIX M. GONI **, DIANA BACH ***, COLIN J. RESTALL and DENNIS CHAPMAN x

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, 8 Hunter Street, London WC1N 1BP (U.K.)

(Received August 23rd, 1979)

Key words: Protein-lipid interaction; Transition temperature; (Ca^{2+} + Mg^{2+})-ATPase

Summary

Differential scanning calorimetry, fluorescence spectroscopy and freeze-fracture electron microscopy have been applied to a study of the reconstituted Ca^{2+}-ATPase proteins from sarcoplasmic reticulum when they are incorporated into pure lipid/water systems. The results obtained with these techniques have been used to examine the effects of this intrinsic protein upon the surrounding lipid at temperatures above and below the main lipid solid-fluid phase transition temperature (T_c).

1. Above this T_c value, the freeze-fracture data show that the proteins are randomly distributed within the plane of the bilayer. The fluorescence data show that as the protein content in the bilayer increases, so does the 'microviscosity'.

2. Below T_c the proteins occur in high protein to lipid patches, separate from the remaining crystalline lipid. The fluorescence data indicate that at these temperatures the presence of the protein causes a decrease in microviscosity, whilst the calorimetric data indicate a decrease in enthalpy of the main lipid transition.

---

* Present address: Departamento de Bioquimica, Facultad de Medicina,Murcia, Spain.

** Permanent address: Departamento de Bioquimica, Facultad de Ciencias, Bilbao, Spain.

*** Permanent address: Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel.

x To whom correspondence should be addressed.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; *P*, fluorescence polarisation defined as $I_{/\!/} - I_{\perp}/I_{/\!/} + I_{\perp}$, in which $I_{/\!/}$ and $I_{\perp}$ are the intensities observed parallel and perpendicular, respectively, to an arbitrary axis; τ, fluorescence excited state lifetime; SDS, sodium dodecyl sulphate; T_c, lipid solid-fluid phase transition temperature.

3. A premelting of the high protein to lipid patches formed by phase separation within the lipid bilayers is indicated by the calorimetric and fluorescence data. This observation is used to rationalise the 'anomalous' properties of the dipalmitoyl phosphatidylcholine-ATPase of exhibiting activity at temperatures well below the lipid phase transition at 41°C.

Introduction

The study of intrinsic protein-lipid interactions is of importance in understanding the dynamics of protein organization and function in biomembrane systems. The knowledge that intrinsic molecules such as cholesterol modulate the lipid bilayer fluidity above and below the main lipid solid-fluid phase transition temperature (T_c) leads [1] directly to questions concerning the extent of the perturbation which intrinsic proteins may cause upon the surrounding lipid. Various authors have recently examined a variety of reconstituted lipid-protein systems, e.g., glycophorin [2], lipophilin [3] or cytochrome oxidase [4]. In previous years, concepts have been developed such as 'boundary layer lipids' and 'annulus lipids'. The first of these concepts was based upon studies of beef heart mitochondrial cytochrome oxidase [5]. Mixtures of the membrane protein with varying amounts of mitochondrial lipid were probed by ESR spectroscopy and the resulting spectra interpreted in terms of a rigid and bulk lipid environment. Studies of reconstituted sarcoplasmic reticulum Ca^{2+}-ATPase in pure lipid/water systems have led to an annulus concept. The intrinsic protein is considered to be present as a complex, i.e., protein and annulus lipids undergoing rotational and lateral diffusion [6] within the lipid bilayer. The dipalmitoyl phosphatidylcholine-ATPase system is also said to be anomalous in that it retains activity well below the main T_c. Here we describe our own recent studies on the perturbation [7] caused by the intrinsic protein, (Ca^{2+} + Mg^{2+})-ATPase, reconstituted into pure synthetic phosphatidylcholines.

Materials and Methods

(Ca^{2+} + Mg^{2+})-ATPase. Sarcoplasmic reticulum was prepared from rabbit back and leg white muscles according to the method of Nakamura et al. [8]. (Ca^{2+} + Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was purified from these membranes according to the method of Warren et al. [9] using 0.5 mg cholate/mg protein. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [10] showed that this protein was more than 95% pure by weight. It was associated with approx. 30 molecules of phospholipid per molecule of ATPase. A molecular weight of 115 000 was assumed for this protein [9].

Lipid : protein recombinants. The purified (Ca^{2+} + Mg^{2+})-ATPase was reconstituted with pure dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine essentially as described by Hesketh et al. [6]. ATPase containing approx. 30 lipids per protein was suspended with sonicated synthetic lipids, dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine (2 mg lipid/mg protein) and sodium cholate (1 mg cholate/mg protein) in a

buffer containing 5 mM Mg^{2+}-ATP, 1 M KCl, 250 mM sucrose, 50 mM Tris-HCl, pH 8 (sucrose buffer). Protein concentration was kept at approx. 10 mg/ml. This mixture was incubated for 30 min at 26—27° C for dimyristoyl phosphatidylcholine and at 42—43° C for dipalmitoyl phosphatidylcholine. Afterwards it was layered on top of a discontinuous sucrose density gradient formed by 1 ml of 50% and 3—4 ml of 15% sucrose in the same buffer as above. These gradients were centrifuged (for 4 h at 250 000 × *g*) at 25° C for dimyristoyl phosphatidylcholine and 38° C for dipalmitoyl phosphatidylcholine. A band of monosubstituted ATPase was recovered and washed in the sucrose buffer. 85—90% of the lipids associated with the protein at this stage was dimyristoyl or dipalmitoyl phosphatidylcholine. A second substitution was carried out as follows: protein (approx. 10 mg/ml) was incubated with lipids (sonicated to clarity in sucrose buffer) and sonicated in a water Soni-cleaner bath (type 6441, Dawe Instrument, Ltd.), for four bursts of 6 min at 26° C for dimyristoyl phosphatidylcholine or 42—42° C for dipalmitoyl phosphatidylcholine.

Cholate was then added and the mixture incubated for 30 min at the same temperature. The amount of lipids ranged from 3 to 5 mg/mg protein and that of cholate from 0.1 to 1.0 mg/mg protein. Using this procedure, it is possible to prepare recombinants of up to 300 phospholipid molecules/molecule of ATPase. We were unable to prepare reconstituted systems with higher lipid : protein ratios, even using as much as 6 mg lipid/mg protein, with 0.09 mg cholate/mg protein. The protein/lipid/cholate mixture was centrifuged in the same gradient and under the same conditions described above when the recombinant was intended to give lipid : protein ratios below 200 : 1. When higher ratios were expected, the suspension was prepared in a 5% sucrose buffer and layered on top of a discontinuous gradient formed by 1 ml 25%, 2 ml 15% and 1 ml 10% sucrose. After centrifugation for 6 h at 250 000 × *g* at the temperatures indicated above, the recombinants were recovered in a band between the 15 and 25% sucrose regions. The recombinants were washed in sucrose buffer in order to remove the residual cholate. According to [^{14}C]cholate measurements, less than 0.2 mol cholate/mol protein was left after three washings followed by sedimentation at 100 000 × *g* for 30 min at 4° C. Gas-liquid chromatography of the recombinant fatty acids showed that at least 99% of the lipids were of exogenous origin. Protein-free dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine samples were prepared by vortex-mixing the lipid together with excess water above its main transition temperature.

Analytical assays. Analysis of the lipid content and composition of the recombinants was achieved by gas-liquid chromatographic determination of their associated fatty acids. The recombinants in aqueous suspension (approx. 50 µl) were evaporated under N_2 at 37° C. Boron trifluoride in methanol (14%, w/v) was added, the tubes closed under N_2 and then heated for 25 min at 90° C. A Pye series 204 gas-liquid chromatograph equipped with a column of 15% poly(ethyleneglycol adipate) on Gas-Chrom Z was used for the analysis. Quantitation was achieved by the use of an internal standard of pentadecanoic acid. Quantitation of lipid phosphorus [11] was also performed occasionally and the data of both procedures were in good agreement (within 10%).

Protein was estimated either according to the method of Lowry et al. [12] or from the ATPase molar absorption, as indicated by Hardwicke and Green

[13]. Dividing the values obtained according to the former method by a factor of 1.2 as described by the latter authors, good agreement was found between both procedures.

ATPase activity was assayed with a regenerating system including 10 mM Mg^{2+}-ATP, 1.5 mM phospho*enol*pyruvate, 0.2 mM NADH, 4 units/ml pyruvate kinase, 6 units/ml lactate dehydrogenase, 100 mM KCl, 5 mM $MgCl_2$, 0.6 mM EGTA and 100 mM triethanolamine hydrochloride, pH 7.2. Samples were equilibrated at the desired temperature before adding $CaCl_2$ up to 0.5 mM.

Chemicals. L-α-1,2-Dipalmitoyl phosphatidylcholine and L-α-1,2-dimyristoyl phosphatidylcholine were obtained from Fluka. ATP for the sucrose buffers was Grade II from Sigma, [^{14}C]cholic acid from the Radiochemical Centre (Amersham, U.K.), diphenylhexatriene and cholic acid from Sigma, and the ATP, enzymes and phosphoenolpyruvate for the ATPase assay from Boehringer-Mannheim.

Freeze-fracture electron microscopy studies. Samples were sedimented, imbibed in 25% glycerol and then equilibrated at the rquired temperature prior to transferring droplets on to gold-nickel specimen holder plates resting on a metal block at the desired quenching temperature. After equilibration for 5 min, the samples were rapidly plunged into partially-solidified Freon 22 and kept under liquid N_2 until fracturing in a Balzers BAF 301 Freeze-Etching Device. Fracturing was carried out at a pressure of approx. $2 \cdot 10^{-6}$ Torr and at −100° C with etching for 30 s. The exposed faces were shadowed with platinum-carbon from a 45° angle, then coated with carbon perpendicular to the specimen stage. Replicas were floated off on water, kept on sodium hypochlorite solution for 1—2 h, washed in water, picked up on untreated 400-mesh copper grids and examined in an AEI-EM6B electron microscope at 60 kV.

Differential scanning calorimetry studies. Calorimetric data were obtained using a Perkin-Elmer DSC2 differential scanning calorimeter. Scanning rates of 5 and 1.25° C/min, and sensitivities of 1 and 0.5 mcal/s were used. Samples were hermetically sealed in Perkin-Elmer aluminium 'volatile' sample pans. The instrument was calibrated with cyclohexane and indium standards. The areas of the peaks were determined by weighing paper cut-outs of the peaks. The phospholipid content of the pans were determined after the measurements by solubilizing the pan contents with 1% SDS at 70° C and analyzing quantitatively the fatty acids present by gas-liquid chromatography with an internal standard of arachidic acid.

Fluorescence polarisation studies. Fluorescence polarisation measurements were carried out using an Elscint MV-1a Microviscosimeter. The temperature of the sample was thermostatically controlled over the range 8—48° C and was measured using a thermocouple inserted into the cuvette. Samples were labelled using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene, at a probe : lipid molar ratio of 1 : 500. Aliquots of a 2 mM solution of the probe in tetrahydrofuran were added to the sample dispersed in sucrose buffer and thoroughly mixed before being incubated for 1 h at 30° C in the case of dimyristoyl phosphatidylcholine and 43° C in the case of dipalmitoyl phosphatidylcholine samples to allow the probe to partition into the lipid. In all cases, the final concentration of tetrahydrofuran was less than 0.001%. To ensure that depolarisation due to light scattering was not occurring, the value of the fluorescence

polarization (P) was measured before and after diluting the sample. In cases where dilution gave an increase in P, the samples were diluted until the value of P had reached a maximum and was no longer concentration-dependent.

Fluorescence excited state lifetime (τ) measurements were carried out on an Applied Photophysics, Ltd. single photon, nanosecond spectrometer, using a nitrogen-filled lamp at 0.5 atm. All the measurements were performed at 36° C.

The general theory of the fluorescence depolarisation measurements applied to membrane systems has been presented in detail [14]. The use of 1,6-diphenyl-1,3,5-hexatriene as a fluorescent probe for the hydrophobic regions of lipid bilayers has also been discussed in detail [15,16].

It has been shown that, using diphenylhexatriene as a probe, P can serve as a relative scale for microviscosity (η) because as the temperature is changed the other factors intervening in the definition of η change much less. The validity of the fluorescence depolarisation technique as a measure of membrane microviscosity has been questioned [17]. The main source of error in the use of P values is a change in τ with increasing concentrations of intrinsic molecules. To check this possibility, τ was measured for recombinants with different lipid : protein ratios. The variation in τ is such that P values are not significantly altered. For instance, τ values for pure dimyristoyl phosphatidylcholine and for a 60 : 1 dimyristoyl phosphatidylcholine-ATPase recombinant are 7.24 and 7.87 ns, respectively. Such increases in the fluorescence lifetime will have the effect of making the observed changes in P slightly smaller than their real values, but not enough to seriously affect the results or their interpretation [14].

Results

Following the cholate-centrifugation method proposed by Hesketh et al. [6] we were able to obtain recombinants of a given lipid : protein ratio, up to 300 mol lipid/mol ATPase. The enzyme activity of such recombinants was approx. 2—3 units/mg at 37° C using dimyristoyl phosphatidylcholine and 3—4 units/mg with dipalmitoyl phosphatidylcholine.

Freeze-fracture electron microscopy

Studies with pure dimyristoyl phosphatidylcholine dispersed in water when quenched from various temperatures show different appearances which are in agreement with previous observations [18].

When protein is incorporated into the dimyristoyl phosphatidylcholine vesicles, different appearances are obtained according to the particular lipid : protein system examined (Fig. 1). With a 237 : 1 ratio, quenched from above T_c, randomly distributed particles are seen among the disordered ridges. When quenched from temperatures between the main T_c and the pre-transition temperature, the particles appear to be present in the form of patches or aggregates and to be excluded from the banded regions [19]. When quenching from below the pre-transition temperature, the ripples disappear but the protein patches remain (Fig. 1).

With the dipalmitoyl phosphatidylcholine recombinant quenched from below its pre-transition temperature similar patches of high protein content were observed.

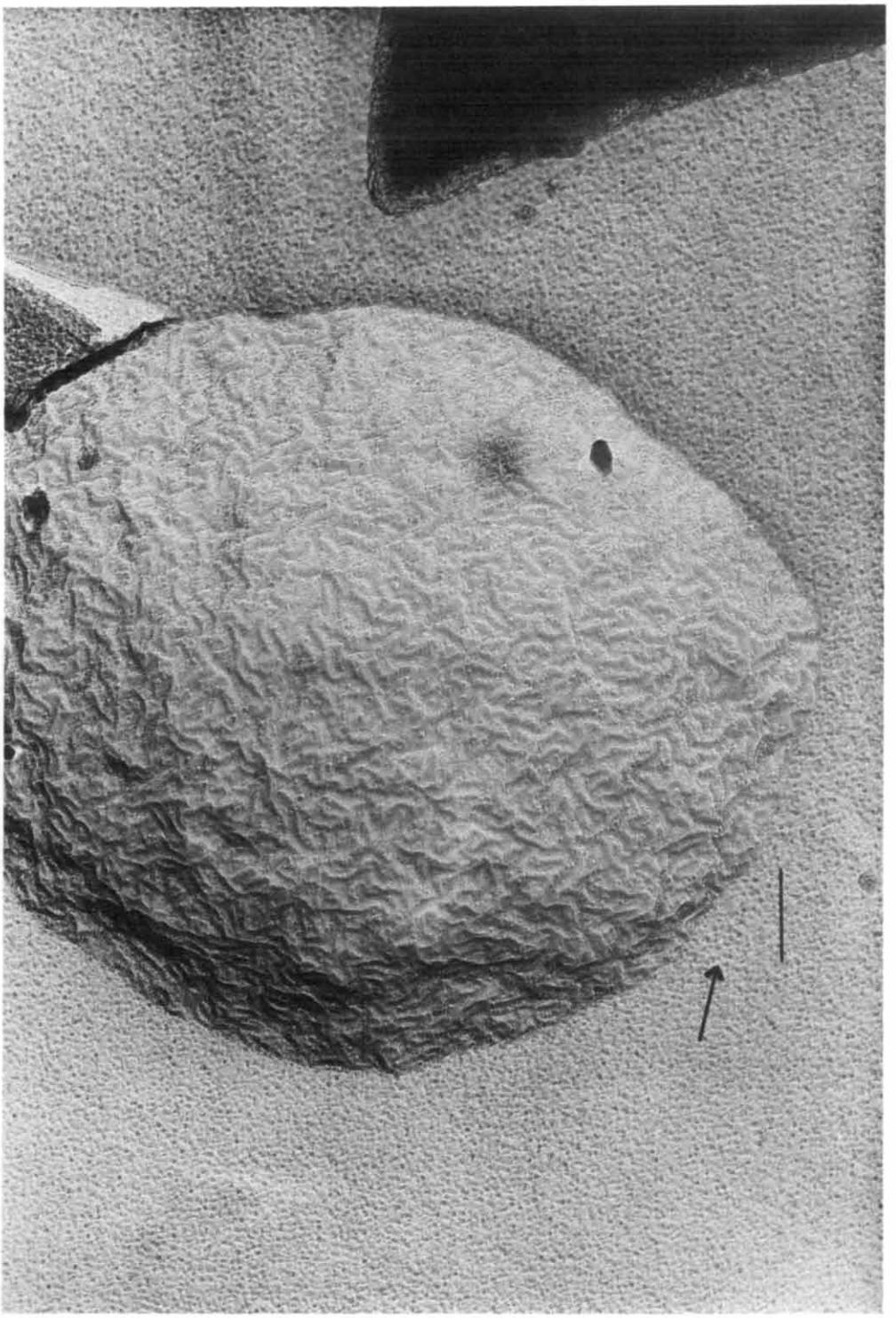

Fig. 1a.

Fig. 1b.

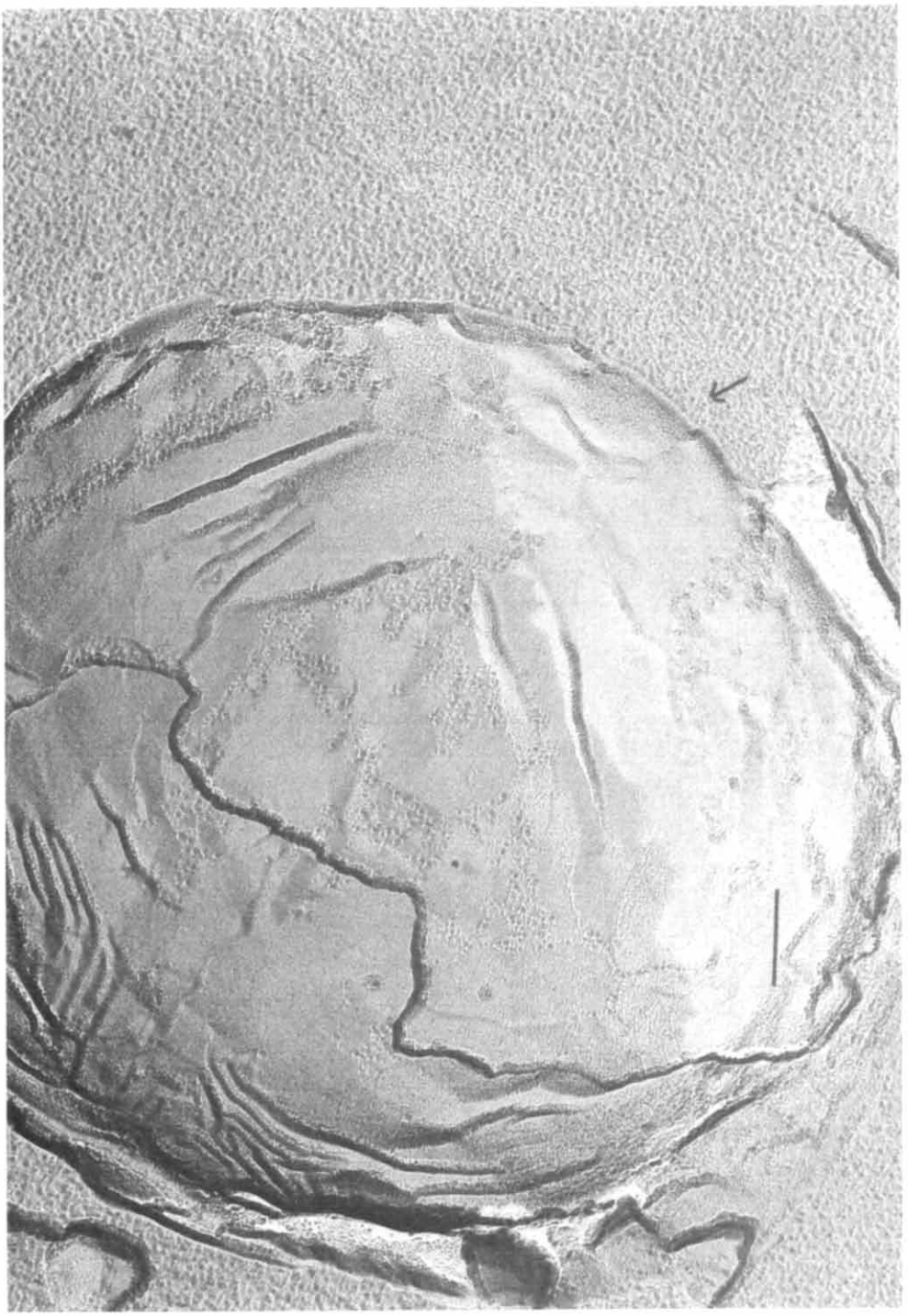

Fig. 1. Freeze-fracture micrographs of dimyristoyl phosphatidylcholine recombinants. The molar ratio of lipid to protein is 237. Sample a was quenched from 37°C, b from 18°C and c from 4°C. The arrows indicate the shadowing directions and the bars represent 0.2 μm .

Calorimetric studies

Differential scanning calorimetric curves of pure dipalmitoyl phosphatidylcholine lipid/water systems (liposomes) and of reconstituted dipalmitoyl phosphatidylcholine-ATPase are shown in Fig. 2. Similar curves for dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylcholine-ATPase have been published [7]. An increase in the protein content causes the pre-transition peak to disappear and the main endotherm to broaden and decrease in size. The midpoint transition temperatures are unchanged within experimental error, whereas the onset temperatures are decreased due to the broadening of the peaks. This behaviour seems to be characteristic of the interaction of phospholipids with intrinsic polypeptides and proteins [20—22].

In addition to the main transition endotherm, a broader transition starting near 30° C, appears superimposed on the former, especially in the protein-rich recombinants (Fig. 2).

When the enthalpy changes associated with the lipid phase transition are plotted against the protein : lipid ratios of the different recombinants, a linear relationship is found (Fig. 3, solid lines). Beyond a given protein : lipid ratio, ΔH becomes too small to be measured from the differential scanning calorimetric thermograms. An extrapolation of the straight lines to $\Delta H = 0$ (Fig. 3, broken lines) allows an estimate to be made of the amount of lipid molecules which are withdrawn from the co-operative transition per molecule of ATPase.

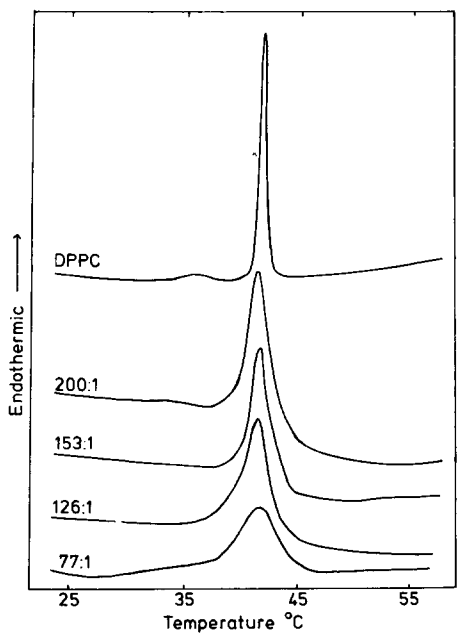

Fig. 2. The calorimetric heating curve for pure dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylcholine-ATPase recombinants. Molar lipid : protein ratios are indicated on the curves. Heating rate, 5°C/min; sensitivity, 1 mcal/s. The curve for pure lipid corresponds to 0.4 μmol dipalmitoyl phosphatidylcholine in the pan. The curves for the recombinants have been normalized to 2.1 μmol dipalmitoyl phosphatidylcholine.

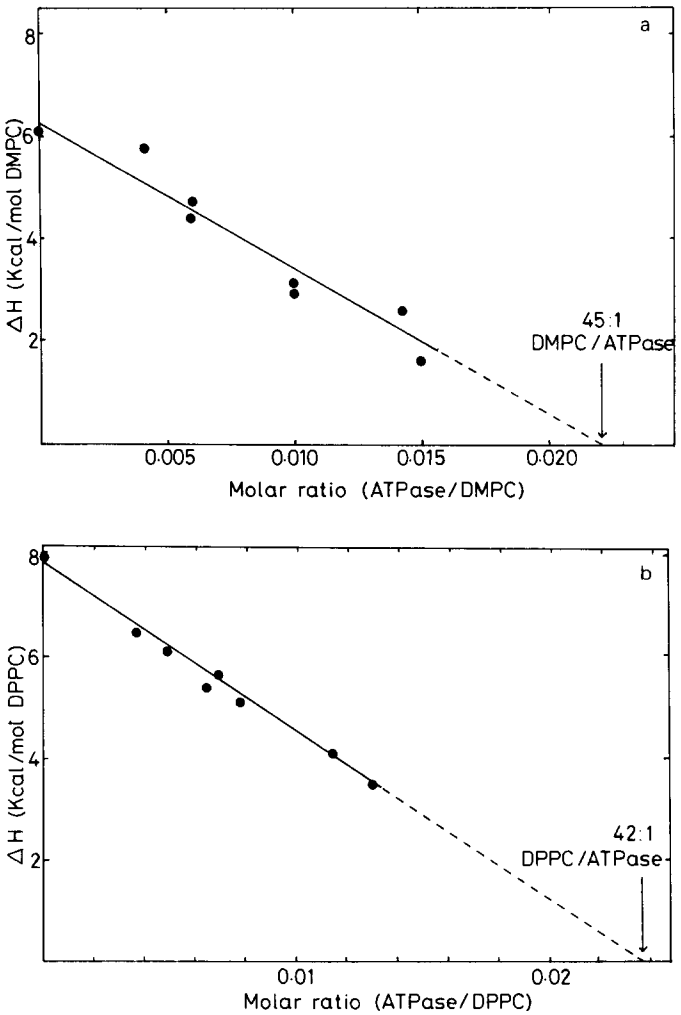

Fig. 3. A plot of the enthalpy change (ΔH) of the main calorimetric endotherm as a function of the molar ratio of ATPase for (a) dimyristoyl phosphatidylcholine (DMPC) and (b) dipalmitoyl phosphatidylcholine (DPPC) recombinants.

Typical figures obtained are 45 dimyristoyl phosphatidylcholine and 42 dipalmitoyl phosphatidylcholine molecules (Fig. 3).

Curatolo et al. [23] were able, but with the use of low scan rates, to distinguish two components in their thermograms of dimyristoyl phosphatidylcholine-myelin proteolipid apoprotein mixtures. In addition to the main component, they found a second partially overlapping higher-temperature melting peak which was attributed to protein-bound lipids. Our scans show that there may be a second peak at high temperature with the dimyristoyl phosphatidylcholine recombinants (see Fig. 2) but this is not certain. Calorimetric measurements were carried out at low scan rates (1.25°C/min) both on dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine-ATPase recombinants, but no better resolution was obtained.

When the calorimetric runs are performed over wider temperature ranges, effects due to protein denaturation are observed. A system with a dipalmitoyl phosphatidylcholine : protein ratio of 80 : 1 was studied in this way. Two denaturation peaks are observed, starting at 59 and 76° C. These peaks are not detected in a subsequent cooling and only a very small peak appears on the second heating run, at 84° C. In this new heating run, the enthalpy change of the main lipid transition is 5.13 kcal/mol dipalmitoyl phosphatidylcholine, whilst the corresponding value from the first scan was 3.42. This corresponds to a 50% increase, but the value corresponding to the pure lipid is not obtained. The protein, even after the heating, is still affecting the lipid. Two possible reasons for this are that total protein denaturation was not achieved under our experimental conditions, or that some of the protein still interacts with the phospholipids.

Fluorescence studies

The fluorescence studies above T_c indicate that the value of P increases as the protein content increases.

The polarisation temperature curves are shown in Fig. 4 for a series of pure lipid and recombinant systems with dipalmitoyl phosphatidylcholine. Similar

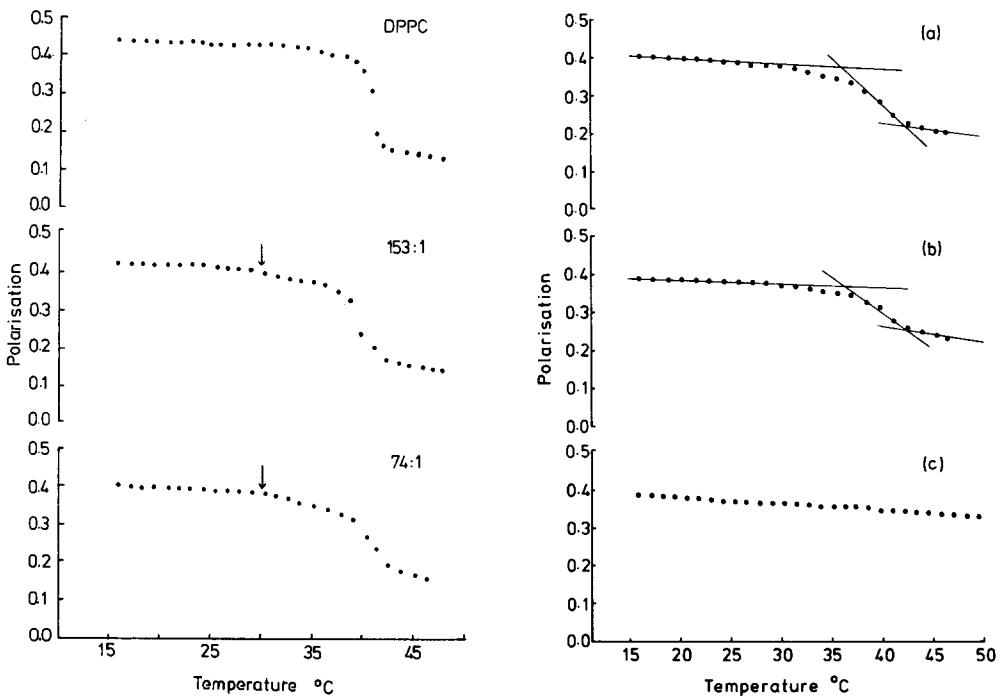

Fig. 4. (a) Polarization (P) values of a fluorescent probe (1,6-diphenyl-1,3,5-hexatriene) in lipid (dipalmitoyl phosphatidylcholine) and lipid-protein recombinants (dipalmitoyl phosphatidylcholine-ATPase) as a function of temperature. The corresponding lipid to protein molar ratios are indicated (the arrows mark 30°C). (b) A comparison of P of the fluorescent probe as a function of temperature for (a) 37 : 1 dipalmitoyl phosphatidylcholine-ATPase recombinant (b) 19 : 1 dipalmitoyl phosphatidylcholine-ATPase recombinant and (c) 1 : 1 dipalmitoyl phosphatidylcholine/cholesterol mixture.

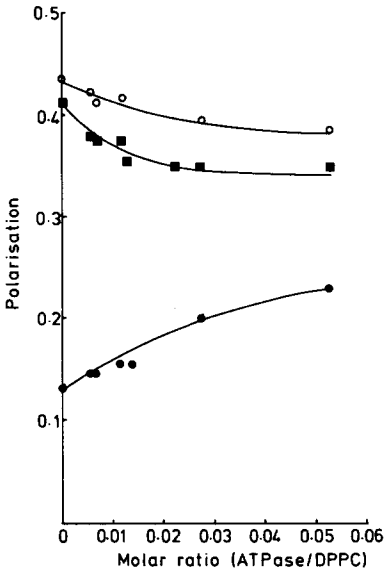

Fig. 5. *P* values of a fluorescent probe in dipalmitoyl phosphatidylcholine-ATPase recombinants of varying lipid : protein ratio at three temperatures (•) 46°C (○) 25°C and (▪) 36°C.

results have been published for dimyristoyl phosphatidylcholine [7]. A general feature below T_c is that as the concentration of the intrinsic protein increases a decrease in the polarisation value occurs. This decrease in polarisation value is less than the corresponding increase which occurs above T_c (see Fig. 5).

When we examine the variation of *P* with temperature (see Fig. 4) for a given protein to lipid ratio (e.g., 196 : 1 for dipalmitoyl phosphatidylcholine recombinant), we see that as the temperature is raised a slow decrease in the value of *P* occurs near 30° C. At this temperature an increased slope of *P* with temperature can be seen. This decrease of polarisation continues until at a higher temperature, near T_c, a sudden further fall in the value of *P* takes place (see Fig. 4). The initial decrease in *P* at approx. 30° C for the dipalmitoyl phosphatidylcholine recombinants appears to be independent of the protein to lipid ratio whilst the second decrease occurs over an increasingly wider temperature range with increased protein content.

A similar effect was observed with the dimyristoyl phosphatidylcholine recombinants where the initial decrease in polarisation value occurred near to 18° C [7].

It can be seen that even at low values of dipalmitoyl phosphatidylcholine to protein (37 : 1 and 19 : 1) a transition still occurs near 41° C (Fig. 4). This can be contrasted with the situation where high amounts of cholesterol are present in a dipalmitoyl phosphatidylcholine system (Fig. 4).

Discussion

The distribution of the $(Ca^{2+} + Mg^{2+})$-ATPase within the sarcoplasmic reticulum membrane has been previously studied by freeze-fracture techniques, and the 8—9-nm particles seen, which probably correspond to proteins embedded in

the membrane, are asymmetrically distributed, most of them being located in the cytoplasmic leaflet [24]. Membrane structures formed by purified ATPase show particles symmetrically distributed in both fracture faces [25,26].

Let us consider the perturbation caused by the intrinsic protein (Ca^{2+} + Mg^{2+})-ATPase above and below T_c.

Above T_c

Our freeze-fracture electron microscope results on the recombinants indicate that above the main T_c [27—29] the proteins are randomly distributed in the plane of the lipid layer. With a pure lipid/water system, e.g., dimyristoyl phosphatidylcholine above the main T_c, the lipid chains are fluid or melted, *gauche* isomers occur in the chains and the lipids can move within the plane of the bilayer [29]. The introduction of a protein into the lipid system can cause some perturbation upon the surrounding fluid lipid [30].

We interpret our fluorescent probe results (Figs. 4 and 5) as indicating that the microviscosity of the lipid increases with the increase of protein content in the lipid bilayer. This result could be interpreted as indicating that the fluidity of the lipid is affected by the presence of the intrinsic proteins and/or that a mere 'mechanical blocking' of movement of the probe occurs. We favour the former conclusion at least until very high protein content is reached. Studies by Cherry et al. [31] with the *Halobacterium halobium* proteins in reconstituted systems have led to similar conclusions. Recent studies using fluorescent probes in delipidated sarcoplasmic reticulum membranes have been given a similar interpretation [32].

Recent evidence has led to doubts about the existence of a long-lived annulus or boundary lipid around intrinsic membrane proteins. Using ^{2}H-NMR spectroscopic techniques, several workers [33—35] have shown that in various protein systems, including (Ca^{2+} + Mg^{2+})-ATPase and cytochrome oxidase, only one type of lipid environment exists, within a time interval of $1 \cdot 10^{-3}$ to $1 \cdot 10^{-4}$ s. This is not compatible with the idea of a long-lived annulus regulating enzyme activity, since the time scale of the latter is one or two orders of magnitude higher. Furthermore, the lipids become more disordered above T_c of the pure lipids, perhaps in order to conform to the irregular surface of the protein [34—36].

Below T_c

The freeze-fracture electron microscopic studies show that, when the recombinants are quenched from below T_c, patches of high protein : lipid ratio occur (see Fig. 1). We consider such patches to be of significance in understanding the biophysical and biochemical properties of these recombinants: (the rippled P_β -phase [27], at certain protein concentrations, can even co-exist with these aggregated patches). This means that below T_c we have to think of formation of patches of high protein to lipid content, precipitating along with any excess crystalline lipid within the lipid bilayer.

The calorimetric data (Fig. 3) show the reduction of enthalpy which occurs with increase of protein content. A reduction of enthalpy per mole of intrinsic protein, in our case equivalent to 45 dimyristoyl phosphatidylcholine molecules per ATPase, has also been observed in other reconstituted systems, such

as gramicidin [22], glycophorin [2] or lipophilin [3]. This could be related to the number of lipid molecules which are in immediate contact with the intrinsic protein. However, this simple explanation may not be satisfactory [37]. To conclude that this enthalpy reduction is immediately a measure of boundary lipid similar to that postulated to occur above T_c (giving rise to ESR immobile components and to the control of enzymatic activity) is, in our view, highly speculative. The fluorescence data indicate that below the main T_c the value of P decreases with increase of protein content in the bilayer (see Fig. 5). Lentz et al. [15,16] suggest that the 1,6-diphenyl-1,3,5-hexatriene probe distributes rapidly and in most cases equally between membrane regions of different composition and fluidity. We rationalise our data in terms of the microviscosity of the protein-lipid patches being lower than that of the crystalline lipid. As more protein is included into the lipid bilayer these patches become larger, the remaining crystalline lipid regions become smaller and the polarisation data become smaller.

The fluorescence data also indicate that a change in polarisation occurs near 30°C prior to the main transition for the dipalmitoyl phosphatidylcholine recombinant, and at 18°C for the dimyristoyl phosphatidylcholine system. As the protein content increases, the widening of the main transition extends until it reaches these temperatures. We interpret these data as indicating that a melting of the high protein-lipid patches occurs followed by the remaining lipid at the main T_c. As the protein content in the bilayer increases further, the amount of excess crystalline lipid becomes smaller and the main transition itself becomes wider. A wide calorimetric transition beginning at approx. 30°C can be seen with the dipalmitoyl phosphatidylcholine system at high protein to lipid content.

Previous workers [6] have suggested that the recombinants of ATPase with dipalmitoyl phosphatidylcholine are anomalous in retaining significant ATPase activity down to 30°C, well below T_c of the pure lipid at 41°C. They attributed this behaviour to the existence of the phospholipid annulus of a single shell of at least 30 lipid molecules.

The possibility that the break in the Arrhenius plot of ATPase activity near 30°C [6] is related to the pre-transition appears unlikely. The pre-transition is removed at protein-lipid ratios of 153 : 1. Our observation of a change of fluorescent probe movement near 30°C with the dipalmitoyl phosphatidylcholine-ATPase recombinants (Fig. 4) is significant. At this temperature we observe also (a) a marked increase in protein rotation using laser flash photolysis and (b) a melting of crystalline hexagonal-packed lipid giving rise to a change of 4.2 Å spacing. We attribute the increase of fluorescent probe, protein movement and enzyme activity at 30°C to a melting of the high protein to lipid patches [38]. This is followed by the melting of the remaining lipid corresponding to the broadened transition near 40°C. Other reconstituted systems may behave in a similar manner.

Acknowledgements

We are grateful to The Wellcome Trust for financial support. J.C.G.F. is a Wellcome Trust Research Fellow. F.M.G. acknowledges financial support from

the 'Patronato de la Universidad de Bilbao'. We thank Dr. Q.F. Ahkong and Dr. A. Brain for assistance with the freeze-fracture electron microscopy, Dr. S. Johnston for assistance with the fluorescence lifetime measurements, Miss M. Turner for help with some differential scanning calorimetry measurements and Dr. P.J. Quinn for useful discussions.

References

1 Chapman, D., Williams, R.M. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445—475
2 Van Zoelen, E.J.J., van Dijck, P.W.M., de Kruijff, B., Verkleij, A.I. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 9—24
3 Boggs, J.M. and Moscarello, M.A. (1978) Biochemistry 17, 5734—5739
4 Marsh, D., Watts, A., Maschke, W. and Knowles, P.F. (1978) Biochem. Biophys. Res. Commun. 81, 397—402
5 Jost, P.D., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 480—484
6 Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K.A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Biochemistry 15, 4145—4151
7 Gomez-Fernandez, J.C., Goni, F.M., Bach, D., Restall, C.J. and Chapman, D. (1979) FEBS Lett. 98, 224—228
8 Nakamura, H., Jilka, R.L., Boland, R. and Martonosi, A.N. (1976) J. Biol. Chem. 251, 5414—5423
9 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 622—626
10 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
11 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—471
12 Lowry, O.H., Rosebrough, N.J., Farr, A.C. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Hardwicke, P.M.D. and Green, N.W. (1974) Eur. J. Biochem. 42, 183—193
14 Shinitzky, M., Dianoux, A.-C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106—2113
15 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528
16 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4529—4537
17 Dale, R.E., Chen, L.A. and Brand, L. (1977) J. Biol. Chem. 252, 7500—7510
18 Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 466, 381—392
19 Kleeman, W. and McConnell, H.M. (1976) Biochim. Biophys. Acta 419, 206—222
20 Chapman, D., Keough, K.M. and Urbina, J. (1974) J. Biol. Chem. 249, 2512—2521
21 Papahadjopoulos, D., Moscarello, M., Eylar, E.H. and Isac, T. (1975) Biochim. Biophys. Acta 401, 317—335
22 Chapman, D., Cornell, B.A., Eliasz, A.W. and Perry, A. (1977) J. Mol. Biol. 113, 517—538
23 Curatolo, W., Sakura, J.D., Small, D.M. and Shipley, G.G. (1977) Biochemistry 16, 2313—2319
24 Deamer, D.W. and Baskin, R.J. (1969) J. Cell. Biol. 42, 183—193
25 McLennan, D.H., Seeman, P., Iles, G.H. and Yip, C.C. (1971) J. Biol. Chem. 246, 2702—2710
26 Packer, L., Mehard, C.W., Meissner, G., Zahler, P. and Fleischer, S. (1974) Biochim. Biophys. Acta 363—159—181
27 Janiak, M.J., Small, D.M. and Shipley, G.G. (1976) Biochemistry 15, 4575—4580
28 Tardieu, A., Luzzatti, V. and Reman, F.C. (1973) J. Mol. Biol. 75, 711—733
29 Chapman, D. (1975) Q. Rev. Biophys. 8, 185—235
30 Curatolo, W., Verma, S.P., Sakura, J.D., Small, D.M., Shipley, G.G. and Wallach, D.F.H. (1978) Biochemistry 17, 1802—1807
31 Cherry, R.J., Muller, V. and Schneider, G. (1977) FEBS Lett. 80, 465—469
32 Moore, B.M., Lentz, B.R. and Meissner, G. (1978) Biochemistry 17, 5248—5255
33 Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H.S., Hshung, J.C., Kang, S.J., King, E., Meadows, M. and Rice, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4657—4660
34 Seelig, A. and Seelig, J. (1978) Hoppe Seyler's Z. Physiol. Chem. 359, 1747—1756
35 Rice, D.M., Meadows, M.D., Scheinman, A.O., Goni, F.M., Gomez, J.C., Moscarello, M.A., Chapman, D. and Oldfield, E. (1979) Biochemistry 18, 5893—5903
36 Chapman, D., Gomez-Fernandez, J.C. and Goni, F.M. (1979) FEBS Lett. 98, 211—223
37 Pink, D.A. and Chapman, D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1542—1546
38 Chapman, D., Cornell, B.A. and Quinn, P.J. (1977) Biochemistry of Membrane Transport, FEBS Symposium No. 42 (Semenza, G. and Carofoli, E., eds.), pp. 72—85, Springer Verlag, Berlin