PROTEIN–LIPID INTERACTIONS

A study of (Ca^{2+}-Mg^{2+})ATPase reconstituted with synthetic phospholipids

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1. Introduction

The presence of intrinsic proteins in biomembranes raises questions concerning the degree and the type of perturbation which these proteins have upon their surrounding lipid environment. Related with these physical effects are the possible consequences of this upon the physiological and biochemical properties of the biomembrane structures.

Only a few physical techniques have as yet been applied to the study of intrinsic proteins in lipid bilayer structures. A technique frequently used has been ESR spectroscopy using spin-labelled molecules. This techniques has been applied to the cytochrome oxidase [1] and sarcoplasmic reticulum ATPase [2] systems. There is, unfortunately, some confusion and controversy concerning the interpretation of these results [3–5]. This is also the case with recent ^2H NMR spectroscopic studies of the reconstituted cytochrome oxidase system [6].

We report here our recent studies on the reconstituted sarcoplasmic reticulum ATPase–lipid–water system, using fluorescent probes and scanning calorimetry. (Our ^2H NMR and other studies will be reported elsewhere.)

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2. Materials and methods

Sarcoplasmic reticulum was prepared from rabbit back and leg white muscles according to [7]. (Ca^{2+}-Mg^{2+})ATPase was purified from these membranes according to [8]. A mol. wt 115 000 is assumed for this protein [8].

The purified (Ca^{2+}-Mg^{2+})ATPase was reconstituted with pure dimyristoylphosphatidylcholine (DMPC) in a similar manner to that in [2], with two susbtitution steps. In the first step, the about 30 mol sarcoplasmic reticulum lipid associated with every ATPase are substituted in a proportion of 80–90% using cholate as equilibrating agent. DMPC (2 mg) cholate (1 mg) per mg protein were suspended and incubated for 30 min at 26°C. The complexes were recovered after sucrose-gradient centrifugation. The second substitution gives ATPase associated with variable amounts of lipids which are >99% DMPC. DMPC and protein are suspended together and, after sonication in a Sonicleaner bath at 26°C for 4 bursts of 6 min, cholate is added and the mixture is incubated for 30 min at 26°C. The amounts ranged from 3–5 mg lipid/mg protein, with cholate from 0.1–1 mg/mg protein. The complexes were recovered after sucrose-gradient centrifugation. According to [^{14}C]cholate measurements, <0.2 mol cholate/mol protein were left after 3 washings, followed by sedimentation at 100 000 × g for 30 min at 4°C.

Calorimetric data were obtained using a Perkin Elmer DSC2 differential scanning calorimeter essentially as in [9]. Scanning rates were of 5°C/min and sensitivities of 1 mcal/s and 0.5 mcal/s. Samples were

hermetically sealed in Perkin Elmer aluminium 'volatile' sample pans. The instrument was calibrated with cyclohexane and indium. The areas of the peaks were determined by weighing paper cutouts of the peaks. The phospholipid content of the pans was 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.

Fluorescence polarisation determinations were made using an Elscint model MV1a Microviscosimeter. Samples were labelled [10] with 1,6-diphenyl-1,3,5-hexatriene (DPH) at a concentration of 1 probe molecule/500 lipid molecules.

Analysis of the lipid content and composition of the complexes was carried out by gas–liquid chromatographic determination of their associated fatty acids. The complexes in aqueous suspension (~50 μl) were evaporated under N_2 at 37°C. Boron trifluoride in methanol (14% w/v) was then added, and the tubes closed under N_2. The samples were then heated for 25 min at 90°C. The gas-chromatographic conditions were as in [11]. Quantitation was achieved by the use of an internal standard of pentadecanoic acid.

Quantitation of lipid phosphorus [12] was also performed, and the data of both procedures were in good agreement. Protein was estimated either according to [13] or from the ATPase molar extinction coefficient, as indicated in [14]. ATPase activity was assayed by the method described in [8] using 10 mM Mg-ATP. Activities of about 3–4 IU/mg at 37°C were found.

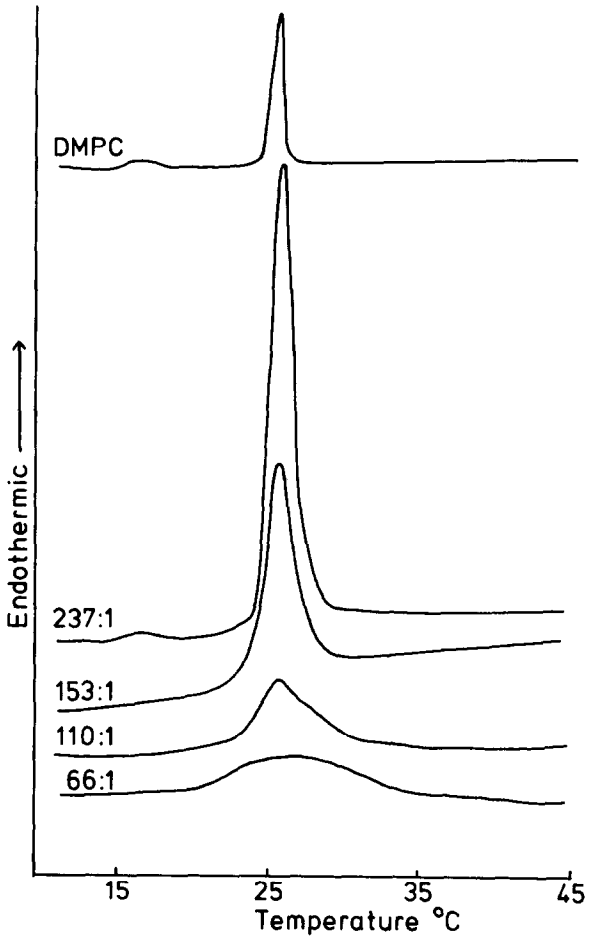

Fig.1. The DSC calorimetric heating curves for pure DMPC and DMPC–ATPase systems. Molar lipid:protein ratios are indicated on the curves. Heating rates: 5°C/min. Sensitivity was 2 mcal/s for the pure lipid and 0.5 mcal/s for protein–lipid systems. The curve for the pure lipid corresponds to 1.0 μmol DMPC in the pan. The recombinant curve have been normalized for 1.4 μmol DMPC in all cases.

3. Results and discussion

Calorimetric DSC studies were carried out on the pure lipid–ATPase systems prepared as described. The effect of the protein on the lipid transition is similar [9] to those observed with gramicidin A. With DMPC, increasing the protein:lipid ratios first removes the pre-transition endotherm and causes the endotherm peak to broaden and to decrease in size (fig.1). The loss of the pre-transition endotherm which accompanied this phenomenon may arise from the protein molecules in the lipid bilayer affecting the existence of the rippled phase and perhaps causing the lipid chains to pack in a vertical configuration [9].

The midpoint transition temperatures are unchanged within experimental error, whereas the onset temperatures are decreased due to the broadening of the peaks. This behaviour appears to be characteristic of phospholipids with integral polypeptides and proteins [15,16] due to a decreased cooperativity of the lipid melting process.

As shown in fig.2 the experiments using the fluorescent probe DPH show results consistent with those

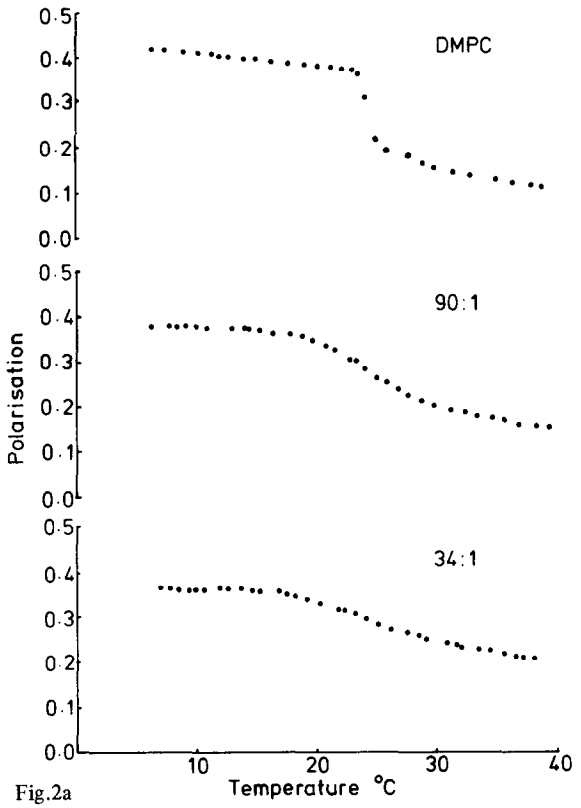

Fig.2a

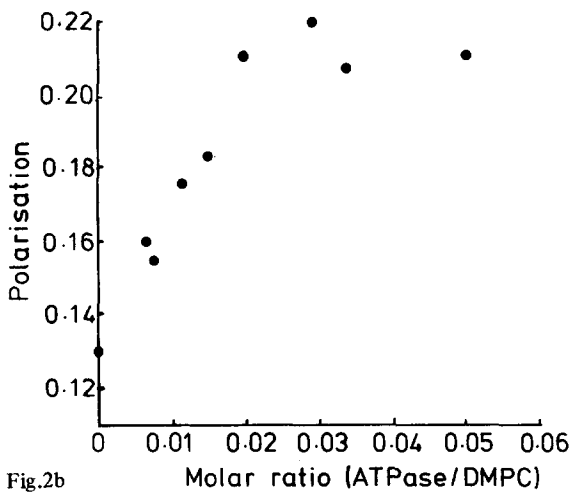

Fig.2b

Fig.2. Polarisation P of a fluorescent probe in lipid and lipid–protein systems containing defined amounts of protein. (a) Polarisation values P at different temperatures. (b) Polarisation values P at a constant temperature (36°C) at various molar protein–lipid ratios.

observed in the scanning calorimetric studies. The presence of protein within the lipid bilayer increases the temperature range in which the change in polarisation ratio occurs.

Proteins appear in the freeze-fracture pictures in the form of particles (to be published). Above the lipid transition temperature T_c randomly distributed particles are observed between the lipid ridges. When the complexes are quenched from below the transition temperature, the particles are excluded from the banded regions, giving rise to aggregated patches. These results are in agreement with those in [17].

At 36°C, well above the transition temperature of the pure dimyristoyllecithin (23°C), the polarisation ratio P increases as the protein concentration increases. This ratio is a measure of the mobility of the probe molecule and has been related [18] to the fluidity or 'microviscosity' of the lipid bilayer. Our observations support the suggestions in [5,19], and require consideration for the interpretation of ESR spin label data in such systems.

Our interpretation of the present physical data is as follows:

The presence of intrinsic protein at low concentration within the lipid bilayer and above the T_c transition temperature will cause a perturbation of the surrounding lipid. This perturbation is probably relatively small in effect, with rapid exchange occurring with the bulk lipid (^{2}H NMR studies to be published). This has been shown to be case for various lipid–protein reconstituted systems [20]. Effects similar to those observed with gramicidin A [9] and also with the myelin apoprotein are expected to occur [22].

Below the T_c transition temperature, as the lipid chains crystallise, the presence of the intrinsic protein creates packing faults [21] and the protein molecules aggregate into high protein–lipid patches (eutectic mixtures) (see fig.3 where this is shown schematically). As yet more protein is included to the lipid system the aggregated patch size increases in size at the expense of the remaining crystalline region. This gives rise to the observed reduction in enthalpy, and the increase of width of the observed transition (fig.1).

Within the aggregated protein–lipid patches the 'microviscosity' is high but will be lower than is the case for a fluorescent probe in the crystalline lipid

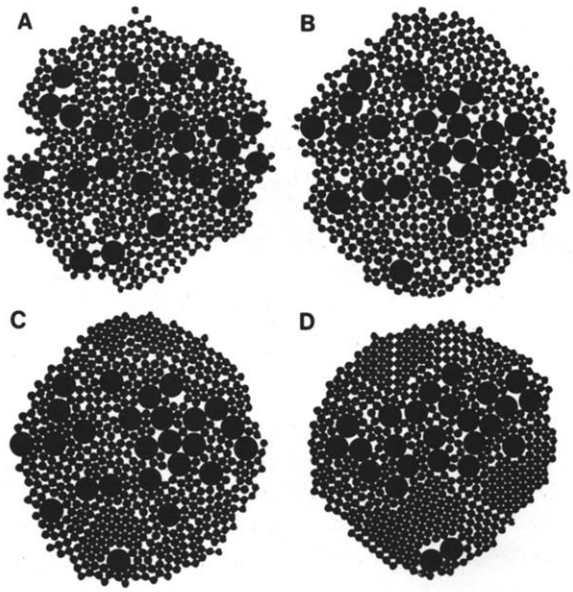

Fig.3. Schematic diagram showing how lipid crystallisation can lead to protein aggregation. (a) Random array of lipid and protein components leading to (b) and (c) nucleation, packing faults and finally in (d) to aggregation of the proteins.

regions (see fig.2a). A spin-labelled fatty acid positioned in this very high protein to lipid region shows an immobile component due to the marked effect of the protein upon this 'trapped lipid', as is observed with gramicidin A [5,9]. When the protein is an enzyme, its motion and enzyme activity may be limited by the highly viscous environment.

As the temperature is raised, the lipids in the aggregated patch (eutectic mixture) can gain mobility, as can a fluorescent label or a spin-labelled molecule or enzyme, at temperatures lower than of the main transition temperature of the pure lipid T_c [17]. However, when there is still sufficient crystalline lipid remaining (free from protein) a disaggregation of the patches will occur at the lipid T_c transition temperature and further effects on fluorescent label (spin label motion) or enzyme activity may occur.

The possibility of protein–protein contacts and eutectic-like patch formation below the pure lipid T_c transition temperature, as well as the protein-induced increase in 'microviscosity' above T_c, have not been considered by previous authors. In our view, however, these phenomena are essential for a proper understanding of the interaction between phospholipids and intrinsic proteins in biomembranes.

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