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PROTEIN-LIPID INTERACTIONS AND DIFFERENTIAL SCANNING CALORIMETRIC STUDIES OF BACTERIORHODOPSIN RECONSTITUTED LIPID-WATER SYSTEMS

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Bacteriorhodopsin has been reconstituted at various molar concentrations into liposomes of dimyristoyl- and also of dipalmitoylphosphatidylcholine. Differential scanning calorimetry indicates that as the protein concentration within the lipid bilayer increases, the cooperativity of the lipid phase transition is reduced, i.e. the transition is broadened, while the midpoint transition temperature remains virtually unchanged. Freeze-fracture electron microscopy of our preparation shows, in agreement with previous data from other laboratories, that extensive protein aggregation occurs when the liposome is cooled below the T_c transition temperature of the lipid. Laser flash photolysis measurements of protein rotation of the bacteriorhodopsin show, especially in the case of protein-rich recombinants, that protein aggregates exist even above T_c. The perturbation caused by the presence of bacteriorhodopsin in the lipid bilayer is similar to that produced by other intrinsic proteins. The difficulty of correlating the observed calorimetric enthalpy data with a simple concept of a 'boundary lipid layer' based upon consideration of a single isolated protein is discussed in view of the occurrence of protein aggregates both above and below T_c. It is concluded that the reduction of enthalpy is related to the number of lipids which solvate the protein aggregates within the protein-lipid patches and are thereby removed from the cooperative melting and enthalpy of the remaining regions of pure lipid.

Introduction

Intrinsic membrane protein-lipid interactions have now been studied in a variety of natural and reconstituted systems [1], e.g. we have recently shown that useful information can be obtained by applying a range of physical techniques, including X-ray, calorimetric and fluorescence methods, to reconstituted Ca^{2+}-ATPase from sarcoplasmic reticulum [2,3]. We emphasised in that study the importance of allowing for the fact that aggregation can occur below the phase transition of the pure lipid/water system. The consequences of this for protein rotation and enzymatic activity below the lipid T_c phase transition were discussed.

Differential scanning calorimetry has often been used to study protein-lipid recombinants and the degree to which the protein can affect the crystalline lipid lattice [1,2,4,5]. However, the interpretation of the calorimetric results is somewhat equivocal; some authors accept the differential scanning calorimetry (DSC) enthalpy data are merely indicating an average number of lipids removed per

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Abbreviations and definition: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; T_c, main transition temperature of the gel-to-liquid crystalline transition of a hydrated phospholipid, or phospholipid/protein recombinant.

protein molecule from the bulk lipid phase to a protein-rich patch [1,2]. Others see in the same data an evidence for the existence of a separate 'boundary layer' of phospholipids [4]. The latter opinion is often supported by the fact that the number of lipids removed from the phase transition is similar, at least in some systems, to the maximum number of lipids that can in principle theoretically contact a single intrinsic protein in a fluid bilayer. The existence of a long-lived ($> 10^{-4}$ s) boundary layer of lipids has been ruled out by ^{2}H-NMR studies in every membrane protein-lipid system studied up to now [6–8], including our own studies of reconstituted bacteriorhodopsin (Chapman, D. and Oldfield, E., unpublished data). The correct interpretation of the calorimetric results depends on various factors. Two sets of data appear to be particularly important in this respect: (i) the size of the protein within the lipid bilayer should be known in order to evaluate correctly the maximum number of lipids which in principle can contact the protein, and (ii) the state of aggregation of the protein under various conditions of protein/lipid ratios and bilayer fluidities should be known so that the ensuing deviations in the number of lipids which may contact each membrane protein might be taken into account. Of the various intrinsic membrane proteins studied up to now, bacteriorhodopsin appears to be the best known in both these aspects, largely due to the efforts of Henderson, Cherry and their co-workers [9–12].

In summary, in the purple membrane bacteriorhodopsin molecules are present in trimers, arranged in a two-dimensional hexagonal lattice. The protein has seven helical residues which extend approximately perpendicular to the plane of the membrane [9]. The cross section of the protein is roughly elliptical, 35×25 Å. When present in DPPC- or DMPC-reconstituted systems well above the T_c transition temperature of the lipid, large protein aggregates are formed at phospholipid/protein molar ratios lower than 50:1, and some aggregation may also be present at higher ratios. Below T_c, extensive protein aggregation is found in all cases [10].

In the present study, we have examined various bacteriorhodopsin reconstituted systems, under conditions leading to different degrees of aggregation. The gel-to-liquid crystalline thermotropic transitions and their associated enthalpies have been evaluated in each case by differential scanning calorimetry. Moreover, the effects of temperature on protein rotation are monitored by laser flash photolysis. The calorimetric data are examined in the light of the known dimensions of bacteriorhodopsin and its state of aggregation.

Materials and Methods

Growth of *Halobacterium halobium* R1 (kindly supplied by Dr. R. Henderson) and purple membrane preparation were performed according to the procedure given by Oesterhelt and Stoeckenius [13]. The lipids used in the experiments were the purest from Fluka and their phase transition behaviour was always checked by calorimetry prior to use.

The method of membrane reconstitution is similar to that described by Hesketh et al. [14] for the Ca^{2+}-ATPase from sarcoplasmic reticulum. Two substitution steps were performed. In the first, purple membrane (10 mg protein/ml) was suspended together with DMPC or DPPC (2 mg/mg protein) in a 150 mM KCl, 20 mM potassium acetate buffer, pH 6, and sodium cholate (2 mg/mg protein). After incubation for 30 min at 27 and 45°C for DMPC and DPPC, respectively, the samples were layered over a continuous sucrose density gradient (15–60% w/v) and centrifuged at $53000 \times g$ for 4 h. The temperature for the centrifugation was 27°C for DMPC and 38°C for DPPC. After centrifugation, the purple band was collected and washed with buffer. For the second substitution step the procedure was repeated, only the lipid and protein mixture was incubated for 20 min in a sonicating bath before the addition of detergent, and the amount of cholate was varied from 1 to 7 mg/mg protein. The recombinants were washed twice with buffer. According to [^{14}C]cholate measurements, less than 0.1 mol cholate/mol protein remained after the washings.

Protein was assayed according to the method of Lowry et al. [15] using bovine serum albumin as standard, or else by the spectrophotometric method described by Rehorek and Heyn [16]. Lipid was assayed according to Bartlett [17] or by quantitative gas-liquid chromatography [2]. The instrument

used was a Pye-Unicam Series 204 gas-chromatograph equipped with a column of 13% (w/w) poly(ethyleneglycol adipate) on Gas Chrom Z.

Samples for freeze-fracture electron microscopy were sedimented, steeped in 25% glycerol, and then equilibrated at the required temperature prior to transferring droplets into silver specimen holders resting on a metal block at the desired quenching temperature. After equilibration for 10 min the samples were rapidly frozen in 'slushed' nitrogen (approx. −210°C). Freeze-fracturing was carried out at −115°C on a Polaron E7500 apparatus. The fracture surface was replicated immediately by shadowing with platinum/carbon and then coating with carbon. The replicas were examined in a Phillips EM 301 G electron microscope.

Calorimetric data were obtained using a Perkin-Elmer DSC2 differential scanning calorimeter. Scanning rates of 5 K/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 analysing quantitatively the fatty acids present by gas-liquid chromatography with an internal standard of arachidic acid. Alternatively, the amount of lipid phosphorus was determined according to Bartlett [17].

For flash photolysis studies, the samples were resuspended in 150 mM KCl, 20 mM acetate (pH 5) buffer, containing 66% glycerol (v/v). The high concentration of glycerol was chosen in order to have no interference from smaller vesicles which might tumble in aqueous suspensions in the time scale of milliseconds.

The flash photolysis apparatus is described elsewhere [18]. Briefly, bacteriorhodopsin is excited by a plane-polarised laser flash. Transient absorbance changes for light parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) with respect to the polarisation of the exciting flash are calculated from the simultaneously measured transmittance changes, $T_{\parallel}$ and $T_{\perp}$. The data are analysed by calculating the anisotropy parameter ($r(t)$, given by $r(t) = A_{\parallel}(t) - A_{\perp}(t)/A_{\parallel}(t) + 2A_{\perp}(t)$. The samples for flash photolysis normally had an absorbance of 0.7–1.5 at the measuring wavelength (570 nm).

In order to test our apparatus for possible artefacts, we prepared bacteriorhodopsin monomers which are rotating in the nanosecond time scale. Purple membrane (approx. 10 mg/ml) was sonicated in the dark for 10 min in a 150 mM KCl, 20 mM potassium acetate buffer, pH 6.0, containing 10% (v/v) Triton X-100 at about 40°C using a bath sonicator (Dawe Instruments Type 6441) in order to solubilize the protein. After centrifugation at $100000 \times g$ for 30 min to remove any residual purple membrane fragments, the supernatant was collected and used without further treatment.

Results

Freeze-fracture electron microscopic observations of our DPPC and DMPC recombinants revealed the same appearance described previously by Cherry et al. [10]: random distribution of protein particles above T_c and particle aggregation, in linear arrays (DMPC) or hexagonal lattice (DPPC, below T_c.

Differential scanning calorimetric curves of pure DMPC and DPPC liposomes and of reconstituted DMPC and DPPC-bacteriorhodopsin are shown in Fig. 1. An increase in the protein content causes first the pretransition peak to disappear and next the main endotherm to broaden and decrease in size. The width of the transition is plotted in Fig. 2, and from 1.5 K for the pure lipid it reaches around 6 K in samples with molar ratios of 20–30 DPPC/bacteriorhodopsin and 5 K in samples of DMPC/bacteriorhodopsin, of the same ratio.

When the enthalpy changes associated with the lipid phase transition are plotted against the protein/lipid ratios of the different systems, a linear relationship is seen (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 removed from the cooperative transition per molecule of bacteriorhodopsin. Typical figures obtained in this way correspond to a decrease of the enthalpy equivalent to 19 di-

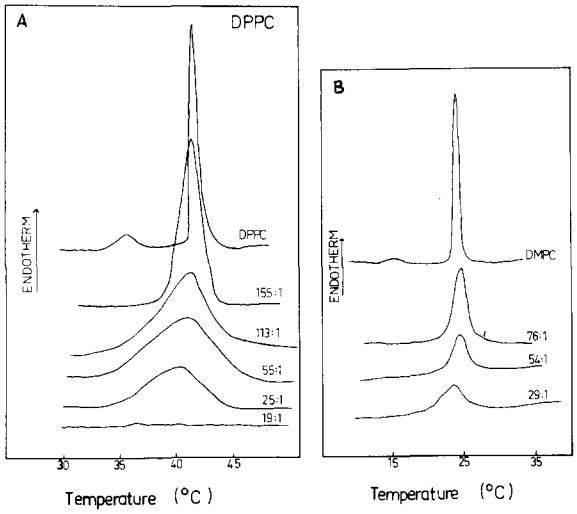

Fig. 1. The calorimetric heating curves for pure dipalmitoylphosphatidylcholine (DPPC) and DPPC-bacteriorhodopsin recombinants (A), and dimyristoylphosphatidylcholine (DMPC) and DMPC-bacteriorhodopsin recombinants (B). Molar lipid/protein ratios are indicated in the curves. Heating rate, 5 K/min; sensitivity, 1 mcal/s. The curve for pure lipid corresponds to 0.87 μmol DPPC and 1.20 μmol DMPC in the pan. The curves for the recombinants have been normalised to 2.91 μmol DPPC and 1.20 μmol DMPC.

myristoylphosphatidylcholine and 22 dipalmitoylphosphatidylcholine molecules. The midpoint transition temperature (T_m) is gradually decreased some 2 K by the incorporation of protein into the lipid bilayer. This behaviour is similar to

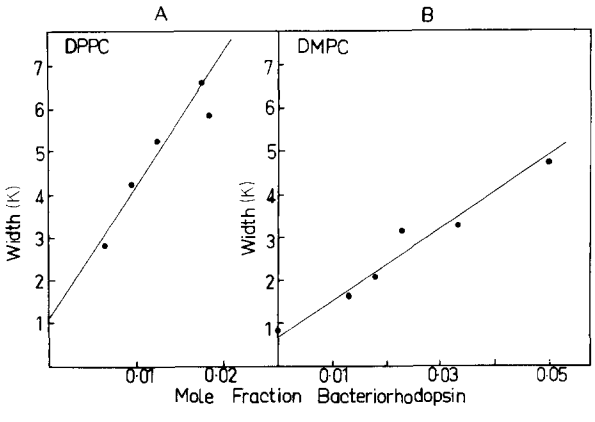

Fig. 2. The width of the transition is plotted against the protein to lipid ratio, for DPPC (A) and DMPC reconstituted samples (B).

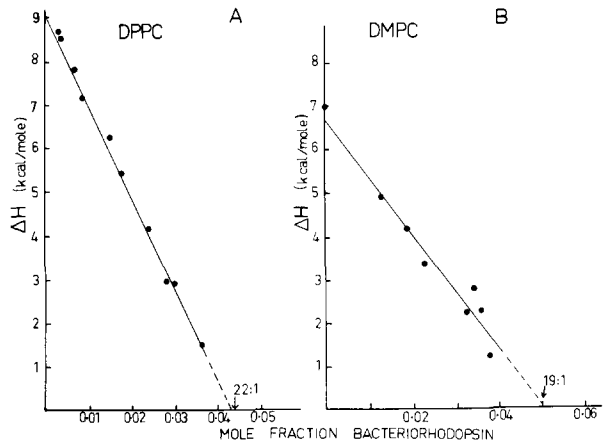

Fig. 3. A plot of the enthalpy change (ΔH) associated with the main calorimetric endotherm as a function of the molar ratio of bacteriorhodopsin incorporated into (A) dipalmitoylphosphatidylcholine (DPPC) and (B) dimyristoylphosphatidylcholine (DMPC).

that observed with other membrane proteins [19,20]. It was observed during our studies that impurities associated with the preparation of the reconstituted system could sometimes lead to marked shifts of T_m to lower temperatures (up to as much as 6 K) and care was required to ensure that protein free from impurities and pure lipids were used in the reconstituted systems.

Time-resolved transient dichroism studies are capable of yielding information about both protein dynamics and the static constraints on the protein movement. In the case of bacteriorhodopsin/phospholipid systems, rapidly rotating protein molecules coexist with protein aggregates too large to rotate within the time-scale of the measurement. These aggregates are more easily found in recombinants with high protein/lipid ratios. It has been shown that [21,22] the fraction of immobile molecules r^{imm}/r_0 can be calculated from the equation:

$$r^{imm}/r_0 = (M^* - M)/(1 - M)$$

where M is the ratio r_∞/r_0 obtained when all the molecules are rotating, i.e. $r^{imm} = 0$ and M^* is the corresponding value for measurements when immobile proteins are expected. From measurements on recombinants of high lipid/protein ratio above the lipid phase transition temperature M was found to have a maximum value of 0.42 for

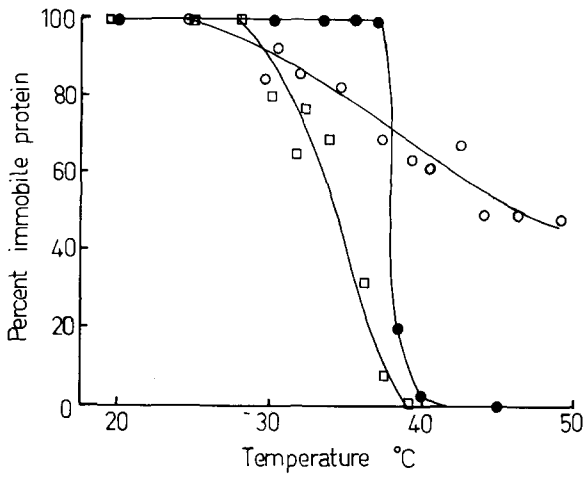

Fig. 4. The percentage of immobile bacteriorhodopsin molecules present deduced from laser flash photolysis studies at various temperatures for three bacteriorhodopsin-DPPC systems. The lipid/protein ratios are (●) 71 : 1, (□) 54 : 1 and (○) 19 : 1.

DPPC/bacteriorhodopsin systems. Using this value, the percentage of immobile proteins over a range of temperatures for a variety of samples has been calculated. The results are shown in Fig. 4 for three DPPC-bacteriorhodopsin recombinants with lipid/protein molar ratios of 71 : 1, 54 : 1 and 19 : 1.

For the 71 : 1 recombinant (filled circles) there is no detectable protein rotation until very near the main transition temperature when the percentage of immobile proteins falls suddenly. At about 42°C, all the proteins are rotating. Increasing the protein content to a molar ratio of 19 : 1 (open circles) causes the onset temperature of protein rotation to be lowered. Protein rotation can be detected at 26°C. Increasing the temperature results in the percentage of immobile proteins decreasing steadily; however, even at 47°C about 50% of the protein remains immobile.

This description of the protein rotation data is useful in this instance as the decay kinetics of $r(t)$ for the lipid-bacteriorhodopsin recombinants shows a distinctive curvature in the semi-logarithmic plot. In many cases at least two and sometimes three separate decay times could be resolved. This complex behaviour makes an accurate quantitative description of the protein rotation data in terms of a rotational motion parameter difficult. Such a multi-exponential decay process is most likely due to bacteriorhodopsin aggregates of various sizes, all of which would be expected to have differing rotational motion parameters.

Discussion

The effect of incorporating bacteriorhodopsin, an intrinsic protein, into the lipid bilayer, is to cause a change of cooperativity of the lipid phase transition. This is demonstrated by the calorimetric data (Fig. 1). No marked shift in the lipid phase transition maximum occurs although in some systems where impurities were present such shifts could occasionally be observed.

Essentially, what takes place is that, when cooling the system, the proteins segregate from the crystallising lipid forming the patches and arrays seen in the electron micrographs [10]. There are, under those conditions, at least two phospholipid populations within the lipid bilayer: the molecules solvating, trapped or associated with the protein patches and those remaining in the regions of pure lipid from which the protein has been excluded. This is in accord with our studies and those of Heyn et al. [12]. Upon raising the temperature of the reconstituted system, in principle at least, two melting processes may occur, the first from the high protein-lipid patches and the second from the remaining lipid. The phase transition and melting process of this remaining lipid will broaden as, with increasing protein concentrations, the patches become larger, i.e. the remaining areas of 'pure' lipid decrease and the cooperativity of the transition also decreases. This explanation fits the observed data showing the broadening of the main transition (Fig. 1).

However, this still leaves the question as to whether a transition should be detectable arising from the 'melting' of the high protein-lipid patches. In an analogous system, the Ca^{2+}-ATPase incorporated into liposomes [3,20], a clear transition was detected by fluorescence probes, calorimetry and X-ray diffraction some 11 K below the main transition found in pure dipalmitoylphosphatidylcholine liposomes. In this case it was shown that the crystalline lipids associated with the patch melted at the lower temperature. The high protein concentration in the patch caused a reduction in the melting temperature of the lipid from 41°C to

30°C. With the bacteriorhodopsin systems no clear transition of this nature was observed. However, bacteriorhodopsin forms much more tightly packed protein-lipid patches. Indeed, a hexagonally packed structure similar to that of the purple membrane appears to form (at least with dipalmitoylphosphatidylcholine) [10]. The lipids in this tightly packed structure will not be able to undergo a cooperative melting process unlike the lipids present in the Ca^{2+}-ATPase protein-lipid patches.

The laser flash photolysis studies demonstrate the existence of varying proportions of immobilised protein as a function of temperature (Fig. 4). These data confirm and complement our interpretation of the ultrastructural and calorimetric observations (a) below the T_c transition temperature of the 71:1 recombinant, all proteins are immobilised, (b) above T_c, a significant proportion of immobilised proteins still exists, especially at high protein/lipid ratios, and the phase transition is broadened and occurs over a wider range of temperature.

Our measurements of the enthalpy variation associated with the thermotropic transitions of the pure lipid and lipid-protein recombinants, show that the equivalent of 22 DPPC and 19 DMPC molecules are removed from the bulk lipid phase per molecule of bacteriorhodopsin incorporated (Fig. 3). Moreover, given the size and shape of the cross-section of the protein [23], a perimeter of about 110 Å can be calculated; this would accommodate 23 alkyl chains, each one occupying 4.8 Å, and taking into account the two phospholipid monolayers making up the membrane, we end with a figure of 23 phospholipids for the maximum amount of these molecules that can contact simultaneously a bacteriorhodopsin monomer.

The coincidence between this theoretical figure and the experimental enthalpy data is interesting, and raises the question as to its meaning. Some workers have interpreted similar data from other systems as indicating the existence of a special boundary lipid layer around each protein. According to this view, each protein molecule would be surrounded, above T_c, by a boundary lipid layer separating it from the bulk lipid phase. Upon cooling, the protein would segregate laterally but with its boundary lipid, the latter not participating in the thermotropic phase transition and therefore decreasing the apparent ΔH of the transition. At least in the case of bacteriorhodopsin, the picture is more complicated, and the above interpreation cannot explain some important facts. (a) In the first place, in the recombinant systems above T_c, the protein occurs in the form of monomers, trimers or even large aggregates, depending on the lipid/protein ratio (Fig. 4) [11]. Since protein-protein contacts occur in these trimers and aggregates [23], the number of lipids which contact each protein is not constant, but decreases with decreasing lipid:protein ratios. (b) Secondly, below T_c, extensive protein aggregation occurs in all recombinant systems. Electron microscopic observations show that, at least in the case of DPPC-recombinants, the protein occurs in a hexagonal lattice, similar to that of the native membrane. In this case it is known that each protein is not surrounded by a lipid shell. (c) Upon consideration of the electron diffraction studies of the native purple membrane, it is clear that, in addition to the lipid in direct contact with the protein, there is a population of phospholipid molecules filling the 'holes' left by the protein trimers in their hexagonal arrangements [23].

We conclude that the reduction of enthalpy observed in this bacteriorhodopsin/lipid system (which occurs as a function of protein concentration) arises in the following way. As the lipid crystallises below its main T_c transition temperature, proteins are excluded from the crystallising lipid (but remain within the plane of the bilayer). The proteins in the form of aggregates take with them solvating lipid and form the high protein-lipid patches. The lipids in these patches make no contribution to the cooperative melting of the lipid chains or to the enthalpy. The remaining enthalpy arises from the residual pure lipid region from which the protein has been excluded. As more protein is included in the lipid bilayer, this patch process continues. The reduction in the number of the lipid molecules available to form the pure lipid region causes a reduction in its cooperativity and also a reduction in the enthalpy of the melting process.

After the experiments reported in this paper were completed, a paper describing similar calorimetric studies was published by Heyn et al. [24]. There is good agreement between our results and

those of the authors mentioned above, and the respective interpretations are also essentially similar.

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