Eur Biophys J (1987) 14: 385–391

European Biophysics Journal

Quantitative analysis of membrane distortions induced by mismatch of protein and lipid hydrophobic thickness

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Received September 9, 1986/Accepted November 7, 1986

Abstract. The phase transitional behaviour of bilayers of the phospholipid L-α-ditridecanoylphosphatidylcholine is studied as a function of protein content for the reaction center (RC) and an antenna protein (LHCP) of the bacterial photosynthetic apparatus. As membrane and protein are structurally well characterized the experimental results can be quantitatively compared with those of calculations based upon elastic models within the Landau-de Gennes-theory. Agreement between theory and experiment demonstrates that dominant elastic forces result from a mismatch of hydrophobic regions of membrane and protein. The data also indicate that RC are present in a monomeric form and LHCP in a highly aggregated form. In addition, the latter protein responds to changes in the lipid environment.

Key words: Protein/lipid interaction, elastic membrane forces, photosynthesis

Introduction

The function of biological membranes depends, to a large extent, on the interaction of membrane proteins with their lipid environment (Singer and Nicolson 1972). This is well recognized but the nature and the origin of the underlying forces are still controversial (Abney and Owicki 1985). To get a better physical picture one has to use proteins of well-known structure and function reconstituted into defined model membranes. These conditions are fulfilled by the systems used in this work, proteins of the bacterial photosynthetic unit incorporated into phospholipid vesicles.

Studies with this model system are also expected to yield information on the structure of the photosynthetic apparatus and, as photosynthesis is a membrane-bound process, on the principles of this fundamental bioenergetic event.

A recent study showed the existence of elastic forces between the photosynthetic reaction center (RC) and the lipid membrane, resulting from a mismatch of hydrophobic regions of lipids and proteins (Riegler and Möhwald 1986). Depending on the lipid chain length the membrane protein shifted the gel/fluid phase transition of the membrane in either direction. In comparing that experimental study with theoretical work a serious drawback resulted from the lack of a quantitative relation between protein concentration and transition temperature shift. This is obviated in the present study using a lipid where

a) relatively large transition temperature shifts are measurable
b) phase transitions occur for conveniently measurable temperatures and where
c) reconstitution without protein degradation is feasible.

We thus show that existing theories and models reasonably well describe the measured elastic forces and beyond that can be used to obtain information on the protein aggregation state. Protein function can in turn be influenced by the lipid environment.

Experimental

Judged from the above criteria lipid selection was performed with the following reasoning:

– The lipid must show a pronounced phase transition, i.e. it must exist of solely one component. The thickness of the hydrophobic part of the membrane should be known. This is the case for diacylphosphatidylcholines with saturated chains (Lewis and Engelman 1983).
– Protein incorporation into the membrane shall have a strong influence on membrane structure. This

is the case for large mismatch between hydrophobic protein and lipid regions. For a given protein of intermediate thickness this calls for lipids with very short or with very long chains.

– As long chain saturated lipids have very high transition temperatures (Silvius et al. 1979) and as reconstitution must be performed while the membrane is in the fluid phase a large protein fraction denatures during incorporation into these membranes. Thus one has to use lipids with short chains, but not as short as to undergo their gel/fluid phase transition below 0 °C.

These arguments recommend use of the phospholipid L-α-ditridecanoylphosphatidylcholine ($D_{13}PC$) with a transition temperature at 13.5 °C in the absence of protein. The lipid (Avanti, Birmingham, Al.) could be used without further purification.

To study elastic interactions a bilayer membrane with low curvature radius was prepared. This was obtained by forming large vesicles by the reverse evaporation technique (Szoka and Papahadjopoulos 1978; Rüppel and Sackmann 1983). Freeze-etch electron microscopy and light microscopy showed that the vesicles prepared had a broad size distribution, but with radii mostly larger than 0.1 μm.

The proteins reconstituted were the reaction center (RC) and the light harvesting chlorophyll protein B 800–850 (LHCP) of the photosynthetic bacterium Rhodopseudomonas sphaeroides (R26 and wild type 241). Bacteria were grown and isolated in the laboratory of Prof. Scheer, LMU Munich, by a procedure modifying that of Jolchine and Reiss-Husson (1974). Their purity and concentration could be checked by absorption spectroscopy. The intensity ratios of the 870, 802 and 280 nm bands of the RC were 1:2:2.3, those of the 850, 800 and 490 nm bands of the LHCP were 2.4:1.3:1. LHCP integrity was assessed by absorption and fluorescence spectroscopy, RC function was measured via absorption changes at 865 nm following excitation by a photographic flash (time duration 1 ms).

The start solution for reconstitution contained 0.025% to 1% LDAO, 0.13 m*M* RC or 0.2 m*M* LHCP. Reconstitution was achieved by mixing the protein solution with lipid vesicles at a temperature above the transition temperature, T_c, and reducing the detergent with dithionite (Pachence et al. 1979) or at a Pb electrode (Riegler et al. 1984). Thin-walled vesicles were prepared by rinsing a buffer solution containing 30 m*M* NaCl, 3 m*M* EDTA and 10 m*M* *Tris* at pH 8 over a thin lipid layer deposited on the glass wall of a flask (Rüppel and Sackmann 1983). The desired amount of protein, typically 0.1 μ*M* was added to the 1 m*M* lipid solution on stirring under nitrogen. After reduction the solution was twice centrifuged (15,000 *g*, 20 min), the lipid/protein sediment (1% of original volume) was then diluted to the original volume. It contained between 20% and 60% of the LHCP or RC input. The protein content of the sample used for measurements was determined by absorption spectroscopy applied on detergent resolubilised RC's.

Lipid phase transition was measured via transmission changes. These reflect the transition as it is accompanied by changes in light scattering. It basically reflects a refractive index change of the membrane (Riegler and Möhwald 1986). The accuracy of temperature measurement was 0.1 °C.

Protein fluorescence was measured using a red-sensitive, cooled photomultiplier (RCA C31034A) and exciting the sample by a slide projector lamp with suitable optical filters. In the fluorescence experiments cuvettes of inner dimensions $3 \cdot 3\,mm^3$ and RC concentrations below 10^{-6} M were used. This ensured that the influence of reabsorption was negligible.

Results

Figure 1 shows freeze-etch electron micrographs of RC (Fig. 1 a) and LHCP (Fig. 1 b) in $D_{13}PC$ vesicles. Dots of uniform size (≈ 100 Å) can clearly be recognized in Fig. 1 a and these can be ascribed to the protein. Obviously there are only few aggregates of RC in this lipid phase. Similar data were also taken for RC in other lipids and phases (Riegler and Möhwald 1986) showing that RC do not aggregate in the fluid lipid phase and in the solid phase for lipid chain lengths between 12 and 15 CH_2 groups.

The situation is much less clear for LHCP in vesicles (Fig. 1 b) where we could not unequivocally localize the protein. This may be due to the fact that the protein of mass 20 kDa is too small to be detected. However, for the following reasons it is more probable that there are aggregates of non-uniform size:

(1) One observes dots of variable size in Fig. 1 b that might be interpreted as protein clusters.
(2) Various optical experiments like singlet exciton annihilation measurements (Grondelle et al. 1983) indicate the existence of LHCP aggregates of about 100 molecules.
(3) Our thermodynamic experiments below also point to the existence of large protein clusters.

The protein influence on the lipid phase transition is given in Fig. 2 for RC in $D_{13}PC$ at variable concentrations. One sees that the sharp phase transition expected at 13.5 °C for the pure lipid (Silvius et al. 1979) can easily be detected by light scattering. Increasing the protein content causes a shift and a

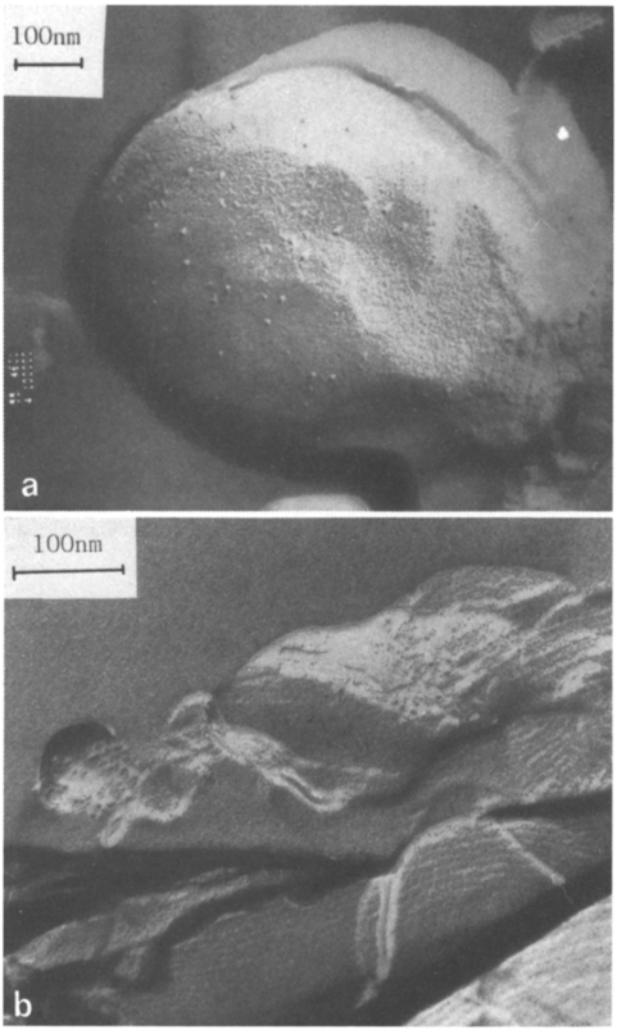

Fig. 1 a and b. Freeze-etch electron micrograph of $D_{13}PC$ vesicles containing RC (**a**) and LHCP (**b**) in a lipid/protein ratio of 1:4,500 and 1:900. Temperature before freezing: 20 °C

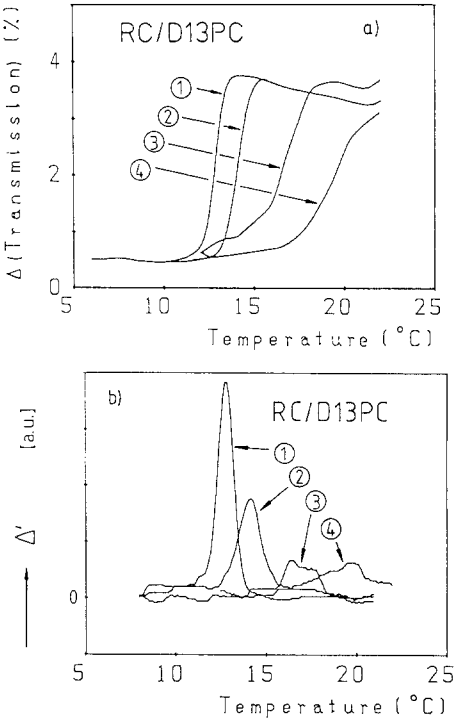

Fig. 2. a Transmission change Δ due to light scattering as a function of temperature for $D_{13}PC$ vesicles containing no RC (trace 1) and RC in a protein/lipid ratio of 1:4,300 (2), 1:2,700 (3) and 1:2,000 (4). 10 m*M* *Tris* buffer, 30 m*M* NaCl, 1 m*M* NaN_3, lipid concentration 10^{-3} *M*. **b** Derivative $\Delta' = \frac{d\Delta}{dT}$ of the curves of **a**

broadening of the transition (Fig. 2a). As the phase change can be measured rather accurately it becomes meaningful to smooth the data and then to record the derivative of the signal with respect to temperature increments. From this one deduces that protein incorporation even in rather small concentrations completely removes the transition of the undistorted membrane. For higher concentrations there are indications of two peaks that would correspond to two populations concerning membrane deformation. We will not comment on this in the discussion. It may reflect a physically interesting feature, but more probably it will be due to vesicle populations containing vastly different amounts of protein. This is also supported by electron microscopic observations (Riegler and Möhwald 1986). For comparison with theoretical calculations the temperatures corresponding to the maxima in Fig. 2a are taken as transition temperatures. In cases where two maxima of similar height were observed, e.g. curve (3) in Fig. 2b, the temperature corresponding to the center of gravity of the line was used. This is somewhat arbitrary but does not affect the conclusions below, as the possible error exists only for high protein content and is below 1 °C.

The linewidth deduced from Fig. 2b is increased by the smoothing procedure from the real value of 0.5 °C to 1 °C in the absence of protein. This procedure therefore hardly affects the curves obtained for protein incorporated. On the other hand the narrow line measured for the pure lipid vesicle in accordance with literature data also reflects material purity and quality of membrane preparation. The broadening and consequently the decrease in peak height with increasing protein content can also be deduced more quantitatively from Fig. 2b.

Qualitatively similar changes of the phase transitional behaviour are observed on reconstituting LHCP into $D_{13}PC$ vesicles (Fig. 3). As with RC there is also an upward shift and a line broadening with increasing protein content. The main difference is that shifts induced by LHCP are an order of magnitude smaller than those due to RC at the same con-

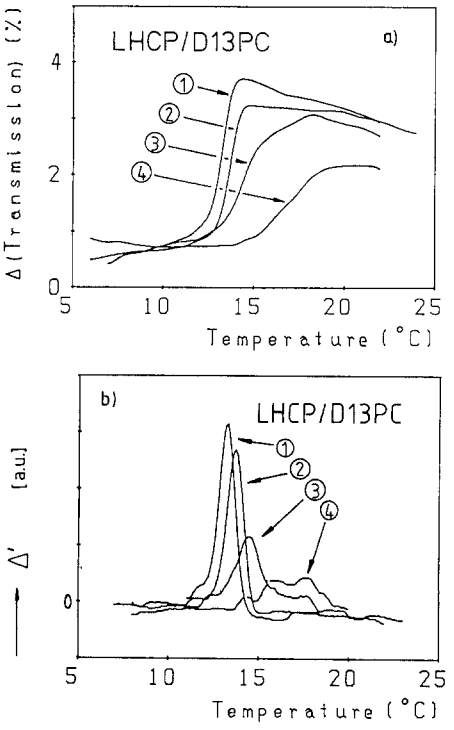

Fig. 3. **a** Transmission change Δ due to light scattering as a function of temperature for $D_{13}PC$ vesicles containing no LHCP and LHCP in a protein/lipid ratio of 1:2,000 (2), 1:1,000 (3) and 1:500 (4). Lipid concentration and buffer solution as in Fig. 2. **b** Derivative $\Delta' = \frac{d\Delta}{dT}$ of the curves of **a**

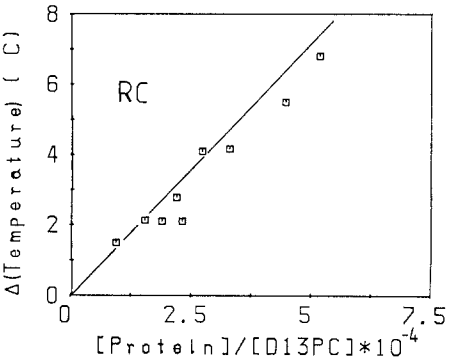

Fig. 4. Shift of transition temperature as a function of protein/lipid ratio for RC in $D_{13}PC$ vesicles under conditions of Fig. 2. The line was calculated for monomeric protein distributions as described in the text

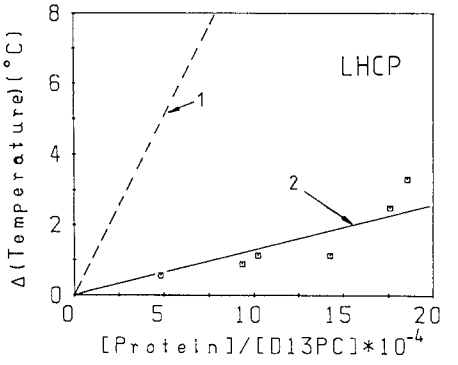

Fig. 5. Shift of transition temperature as a function of protein/lipid ratio for LHCP in $D_{13}PC$ vesicles under conditions of Fig. 3. The lines were calculated as described in the text assuming a monomeric protein distribution (line 1) or two-dimensional LHPC aggregates of average size 50 molecules (line 2)

centrations. In the discussion we will show that this is basically due to LHCP aggregation. The shift of transition temperature as a function of RC and LHCP concentration is given in Figs. 4 and 5, respectively. These shifts are roughly linear in protein content and can be compared with those calculated from extending the theoretical model as described in the discussion.

The change of the lipid environment does not affect RC function judged from the magnitude and time constant (100 ms) of the absorbance recovery at 865 nm after flash excitation. This measurement reflects the back transfer of an electron from the primary quinone to the donor site (Clayton 1980). LHCP function, however, is changed during the transition as shown in Fig. 6. Although the shape of the emission spectrum is independent of temperature (and also excitation wavelength), the fluorescence intensity increases by up to a factor of two on going from the solid to the fluid phase. The change is reversible and supposedly due to a variation in the protein aggregation state. The most interesting feature to be discussed below as due to a premelting phenomenon is the finding that the "fluorescence detected transition" occurs at a somewhat lower ($\approx 1\,°C$) temperature than the transition detected by light scattering. Additionally transitions detected by both techniques are shifted in parallel with protein content (compare Fig. 6 a and b).

Discussion and conclusions

The experimentally observed shift of transition temperature with protein content can be described basically using the Landau-de Gennes theoretical approach (Owicki and McConnell 1978, 1979; Jähnig 1981). The free energy density f is given as a power series of an order parameter S plus an elastic term describing local variation of the order parameter (Jähnig 1981) (1)

$$f = \tfrac{1}{2} a_2 (T - T^*)\, S^2 - \tfrac{1}{3} a_3 S^3 + \tfrac{1}{4} a_4 S^4 + \tfrac{1}{2} b \left(\frac{\partial S(r)}{\partial r} \right)^2$$

a_2, a_3, a_4, and b are temperature and space independent parameters, T^* is a temperature close to the

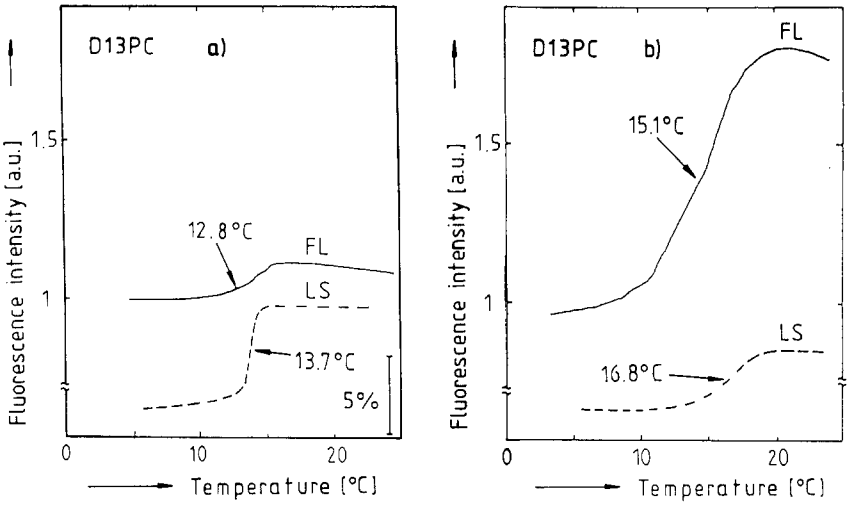

Fig. 6. Transmission change due to light scattering (*LS*) and fluorescence intensity at 860 nm (*FL*) as a function of temperature for LHCP in $D_{13}PC$ vesicles in a protein/lipid ratio of 1:5,000 (**a**) and 1:500 (**b**). Lipid concentration and buffer solution as in Fig. 2

transition temperature and r is a distance from the center of the protein. Since we want to show that the main elastic force results from an expansion or a compression of the membrane by the protein in normal direction, we use S as the thickness of the hydrophobic part of the membrane (Mouritsen and Bloom 1984). Hence we assume that the main membrane distortion results from the mismatch of hydrophobic membrane and protein parts. This is supported by previous observations (Riegler and Möhwald 1986) showing an increase or decrease of the transition temperature on RC incorporation, depending on the membrane thickness.

In normalized units we define

$$S = \frac{d_f - d(r)}{d_f - d_s}, \tag{2}$$

where d_f and d_s are the thickness of the undistorted fluid and solid membrane, respectively and where $d(r)$ is the (local) membrane thickness.

The protein in the membrane creates a local order parameter variation decaying exponentially with a characteristic decay length ξ_i.

$$S(r) = S_u + (S_0 - S_u) \exp(-(r - R_0)/\xi_i) \tag{3}$$

(S_u, S_0 = order parameters of the undistorted membrane and at the membrane/protein interface. Minimizing the total free energy $F = \int f\, dr$ one then obtains a relation between a shift of the transition temperature ΔT and the mean distance $2R$ between two proteins

$$\Delta T = \frac{4b}{a_2 (R^2 - R_0^2)} \left(2\frac{R_0}{\xi_i} + 1\right)\left(2\frac{S_0}{S_u^g} - 1\right) \tag{4}$$

(S_u^g = order parameter of the undistorted membrane in the gel phase). With a mean lipid area A of 33 Å^2 (Lewis and Engelman 1983) R can be calculated from the protein/lipid ratio P according to

$$R^2 - R_0^2 = \frac{A}{\pi \cdot P} = \frac{10\,\text{Å}^2}{P}. \tag{5}$$

Hence from Eq. (4) follows the linear relation between ΔT and P:

$$\Delta T = \frac{4b}{a_2 \cdot 10\,\text{Å}^2}\left(2\frac{R_0}{\xi_i} + 1\right)\left(2\frac{S_0}{S_u^g} - 1\right) \cdot P. \tag{6}$$

To estimate the proportionality constant Jähnig (1981) used $b/\xi_i^2 \cdot a_2 = 20\,°\text{C}$ from theoretical arguments and assumed $\xi_i = 15$ Å.

The membrane thickness is derived from interpolation of X-ray diffraction data (Lewis and Engelman 1983) on $D_{12}PC$ and $D_{14}PC$ as $d^f = 21$ Å. One may assume the gel phase membrane to be about 30% thicker, hence $d^g = 27$ Å (Janiak et al. 1976).

The thickness of the hydrophobic part of the RC has previously been determined as 28 Å (Riegler and Möhwald 1986) in accordance with data on the amino acid sequences of the two subunits L and M spanning the membrane (Williams et al. 1983, 1984), the RC diameter may be taken as $2R_0 = 60$ Å.

The LHCP hydrophobic thickness is taken as 31 Å from X-ray work (Zuber 1985). As the monomeric protein unit consists merely of two transmembrane helices we assume R_0 (LHCP) = 12 Å.

With these values one obtains from Eq. (6):

$$\Delta T = P \cdot 1.4 \cdot 10^4\,[°\text{C}] \quad \text{for RC} \tag{7a}$$

$$\Delta T = P \cdot 1.1 \cdot 10^4\,[°\text{C}] \quad \text{for LHCP}. \tag{7b}$$

The calculated linear relations for the monomeric protein can be compared with experimental data in Figs. 4 and 5. For RC the agreement between theory and experiment is surprisingly good in view of the many simplifications used in deriving Eqs. (7). This indicates that values of ξ_i and of b are rather good

estimates and that neglecting phase separations is not that critical when considering "mean" transition temperatures.

For LHCP on the other hand there are discrepancies of about an order of magnitude which may be understood as arising from protein aggregation with the following argument: If N proteins form a two-dimensional aggregate the boundary with the lipid will decrease by a factor of $\sqrt{N}$ per protein. This effectively reduces the distorted lipid area and consequently has a smaller influence on the transition temperature.

Assuming average LHCP aggregate sizes of 50 molecules one thus derives the linear relation given for an aggregate in Fig. 5. Obviously in view of the many assumptions involved comparison between theory and experiment is not a good measure of N. On the other hand the value of N needed to simulate the experimental data is not unrealistic in view of independent optical experiments (Grondelle et al. 1983).

Our experiments also indicate that the aggregate is more two-dimensional than one-dimensional. For the latter case one can easily see that aggregation reduces the protein/lipid boundary by at most 40% and thus cannot have an influence of a factor of ten on temperature shifts, as experimentally observed (Peschke 1986).

Hence we can show that for two membrane proteins at known concentration in the bilayer there is good quantitative agreement between experiment and calculations based upon previously developed theories and parameter estimates in combination with known structural parameters of membrane and protein. These results are presumably much more general because the two proteins are structurally very different. (*i*): The RC (molecular weight ≈ 100 kDa) exhibits 11 transmembrane helices (Deisenhofer et al. 1984) whereas LHCP (molecular weight ≈ 18 kDa) exhibits only two. (*ii*): The hydrophobic thickness of RC is 28 Å, that of LHCP 31 Å. (*iii*): RC are embedded into the membrane in a monomeric form, LHCP in aggregate form. (*iv*): RC function is independent of membrane environment whereas LHCP fluorescence is not. Nevertheless both proteins induce an increase in transition temperature which also means that the order they induce by stretching the membrane dominates other forces creating disorder.

These other forces are of course present as the protein surface, which is surely rough, will induce defect formation in the adjacent hydrocarbon chains. This would cause a reduction of the transition temperature in contrast to the experimental findings. On the other hand we deduce from fluorescence experiments (Fig. 6) that on approaching the phase transition the protein environment becomes fluid more quickly than the remainder of the membrane. This would not be expected in a model which only accounts for hydrophobic mismatch that in turn is smaller in the gel phase. It indicates that there are forces dominating at different distances from the protein. In its immediate boundary the protein favours chain disorder whereas the tendency to stretch the membrane persists over several lattice constants, the latter in agreement with a statistical mechanical calculation (Marcelja 1976). The long range nature of the distortion is also indicated from the observation that even at low protein content (e.g. $P = 1/2{,}000$) we observe only one shifted phase transition and not two transitions. The latter would be expected if in the experiment we had to distinguish between undistorted and distorted membrane regions.

We should also point out that we observed the broadening of the phase transition as theoretically expected for protein reconstitution (Jähnig 1981). We did, however, not analyse this in detail because it is very sensitive to preparation conditions resulting in different protein concentrations in different vesicles. These variations have little influence when considering the (mean) transition temperature for concentrations where the linear relation (Eq. (7)) holds.

Finally, we would like to comment on the biological significance these experiments might have, despite being performed with a highly artificial system. If the biological membrane were built up of saturated lipids with aliphatic chain lengths equivalent to the hydrophobic thickness of the proteins it would be in the gel phase at room temperature. To prevent this nature uses mixtures of mostly unsaturated lipids. There phase transitions are difficult to detect.

Experimental and theoretical study of a phase transition can on the other hand be regarded as shedding light on features that are amplified by cooperative forces modulating the transition. Thus one may state that elastic forces described in this work are more difficult to detect in a complicated lipid alloy but are surely present. Therefore it is highly desirable to extend these studies with well-defined lipids and proteins to obtain a more microscopic picture of the protein environment in the membrane.

Acknowledgements. We appreciate the criticism of one of the referees of our paper (Riegler and Möhwald 1986) bringing to our attention the availability of $D_{13}PC$ and acknowledge valuable advice and protein supply by H. Scheer. We also profited from discussions with E. Sackmann and the technical assistance of C. Fahn. The work was supported by the Deutsche Forschungsgemeinschaft through SFB 143/A4.

References

Abney JR, Owicki JC (1985) Theory of protein-lipid interactions. In: Watts A, De Pont JJHHM (eds) Progress protein-lipid interactions, vol 1. Elsevier, Amsterdam

Clayton RK (1980) Photosynthesis: physical mechanisms and chemical patterns. JUPAB Biophysics. Cambridge University Press, New York

Deisenhofer J, Epp O, Miki K, Huber R, Michel H (1984) X-ray structure analysis of a membrane protein complex. J Mol Biol 180: 385–398

Grondelle RV, Hunter CN, Bakker JGC, Kramer HJM (1983) Size and structure of antenna complexes of photosynthetic bacteria as studied by singlet-singlet quenching of the bacteriochlorophyll fluorescence yield. Biochim Biophys Acta 723: 30–36

Jähnig F (1981) Critical effects from lipid-protein interaction in membranes. I. Theoretical description. Biophys J 36: 329–345

Janiak MJ, Small DM, Shipley GG (1976) Nature of the thermal pretransition of synthetic phospholipids: Dimyristoyl- and dipalmitoyllecithin. Biochemistry 15: 4575–4580

Jolchine G, Reiss-Husson F (1974) Comparative studies on two reaction center preparations from Rhodopseudomonas sphaeroides Y. FEBS Lett 40: 5–8

Lewis BA, Engelman DM (1983) Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. J Mol Biol 166: 211–217

Mouritsen OG, Bloom M (1984) Mattress model of lipid-protein interactions in membranes. Biophys J 46: 141–153

Owicki JC, McConnell HM (1978) Theoretical study of protein-lipid interactions in bilayer membranes. Proc Natl Acad Sci USA 75: 1616–1619

Owicki JC, McConnell HM (1979) Theory of protein-lipid and protein-protein interactions in bilayer membranes. Proc Natl Acad Sci USA 76: 4750–4754

Pachence JM, Dutton PL, Blasie JK (1979) Structural studies on reconstituted reaction center-phosphatidylcholine membranes. Biochim Biophys Acta 548: 348–373

Peschke J (1986) Protein/Lipid-Wechselwirkungen von Proteinen des Photosyntheseapparates in und an Modellmembranen. Thesis, TU Munich

Riegler J, Möhwald H (1986): Elastic interactions of photosynthetic reaction center proteins affecting phase transitions and protein distributions. Biophys J 49: 1111–1118

Riegler J, Peschke J, Möhwald H (1984) Two-dimensional electron transfer from cytochrome c to photosynthetic reaction centers. Biochem Biophys Res Commun 125: 592–599

Rüppel D, Sackmann E (1983) On defects in different phases of two-dimensional lipid bilayers. J Phys (Paris) 44: 1025–1034

Silvius JR, Read BD, McElhaney RN (1979) Thermotropic phase transitions of phosphatidylcholines with odd-numbered n-acyl chains. Biochim Biophys Acta 555: 175–178

Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 173: 720–731

Szoka F, Papahadjopoulos D (1978) Procedure for preparation of liposomes with large internal aqueous space capture by reverse-phase evaporation. Proc Natl Acad Sci USA 75: 4194–4198

Williams JC, Steiner LA, Ogden RC, Simon MI, Feher G (1983) Primary structure of the *M* subunit of the reaction center from Rhodopseudomonas sphaeroides. Proc Natl Acad Sci USA 80: 6505–6509

Williams JC, Steiner LA, Feher G, Simon MI (1984) Primary structure of the *L* subunit of the reaction center from Rhodopseudomonas sphaeroides. Proc Natl Acad Sci USA 81: 7303–7307

Zuber H (1985) Structure of antenna peptides. In: Antenna and reaction centers of photosynthetic bacteria. Springer Series in Chem Physics, vol 42. Springer, Berlin Heidelberg New York, pp 2–14