Lipid Influence on the Structure of the Light Harvesting B 800–850 Proteins

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Interaction of the antenna protein B 800–850 with the lipid environment and with the reaction center of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* is studied by fluorescence spectroscopy, transient absorption techniques, light scattering and electron microscopy. Using vesicles of synthetic phospholipids it is shown that solidification of the membrane causes a structural protein change evident from a reduction in fluorescence quantum yield. The change occurs at a temperature up to 5 °C below that corresponding to the gel/fluid transition temperature and indicates local melting. The structural change is not specific for the lipid head group nor chain length (investigated for lengths of 12 to 16 CH_2 groups) and can be understood applying a simple elastic model. It can also be induced isothermally by changing the ionic milieu and thus varying the lipid phase state.

Energy transfer LHCP→RC is proven to be highly efficient in model membranes and is not affected by the existence of a phase transition. This indicates two LHCP fractions one tightly – and one non-bound to the RC.

Introduction

Proteins from the photosynthetic apparatus are well-suited to study protein/lipid interactions as they are structurally well-known and can be characterized functionally by optical [1] and by magnetic resonance techniques [2]. They can therefore serve to investigate the general physical principles of structure/function relationship in biological membranes. This relationship is important to understand photosynthesis as a membrane-bound process. It is obvious that the organization may affect functions like energy, electron or proton transport but it is also possible and will be demonstrated in this work that protein internal processes like vibrational relaxation may be influenced by the membrane environment.

Interactions may result from electrostatic and elastic forces [3] and can be assessed reconstituting proteins into well-defined membranes. Elegant reconstitution techniques have been developed but were basically applied on membranes of natural lipids [4, 5]. These membranes containing mixtures of lipids and existing only in the fluid state for temperatures above 0 °C do not allow to assess the influence of the lipid environment as regards to hydrocarbon chain length, head group and phase state. Therefore we reconstituted reaction centers and/or the antenna protein B 800–850 of the photosynthetic bacterium *R. sphaeroides* into vesicles [6] and monolayers of synthetic phospholipids [7]. Studying lipid phase transitions, protein distributions and protein functions we assessed influences on lipids as well as on proteins. We could show in how far proteins affect the lipid environment in terms of ordering. Proceeding along this line we will demonstrate that the lipid environment affects the LHCP and provide information in how far energy transfer processes are altered by this.

Materials and Methods

RC and LHCP of *Rhodopseudomonas sphaeroides* (R 26 and wild type 241) were grown and isolated according to a procedure modifying that of Jolchine and Reiss-Housson [8]. Purity was checked by absorption spectroscopy. The start solution for reconstitution contained 0.025% to 0.1% LDAO, 0.13 mM RC or 0.2 mM LHCP. The lipid DLPC was from Fluka, Buchs, CH, DMPC, DPPC and DLPA were

Abbreviations: R. sphaeroides, Rhodopseudomonas sphaeroides; RC, reaction center; LHCP, bacterial antenna protein B 800–850 (light harvesting chlorophyll protein); DLPA; L-α-dilauroylphosphatidic acid; DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; LDAO, N,N-dimethyldodecylamin-N-oxid (lauryldimethylamin-oxid); BChl, bacteriochlorophyll *a*; EDTA, ethylenediamine-tetraacetic acid (tetrasodium salt).

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from Sigma, Munich and used without further purification. All lipids were chromatographically checked to be 99% pure. NaCl, EDTA and Tris-HCl were of PA-standard.

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 [5]. Vesicles were prepared by rinsing a buffer solution containing 30 mM NaCl, 3 mM EDTA and 10 mM Tris at pH 8 over a thin lipid layer deposited on the glass wall of a flask [9]. By light microscopy we checked for each preparation that vesicles had a mean size of about 2 μm with a distribution of sizes between 0.5 μm and 5 μm. This was also confirmed by freeze-etch electron micrographs taken for selected samples by the method of ref. [9]. Electron microscopy showed that only a small fraction of lipids was in vesicles of sizes smaller than 0.5 μm and also that the vesicles were predominantly unilamellar. The desired amount of protein, typically, 0.1 μM was added to the 1 mM lipid solution on stirring under nitrogen. After reduction the solution was twice centrifuged ($15000 \times g$, 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 and purity of the sample used for measurements were determined by absorption spectroscopy applied on detergent resolubilised proteins. The lipid content was assessed by a modified phosphate determination [10]. The integrity of proteins reconstituted into vesicles was checked by measurements of absorption and fluorescence spectra for LHCP and of stationary and transient absorption changes for RC. Samples for experiments with vesicles containing both types of proteins, one in constant, the other in variable concentration were prepared by first reconstituting one protein then splitting the sample and incorporating the second protein in varying amount.

Lipid phase transition was measured via transmission changes. These reflect the transition as it is accompanied by changes in light scattering [6]. Protein fluorescence was measured using a red-sensitive, cooled photomultiplier (RCA C31034A) and exciting the sample by a slide projector lamp and suitable optical filters. In the fluorescence experiments cuvettes of inner dimensions 3*3 mm^2 and RC concentrations below 10^{-6} M were used. This ensured that the influence of reabsorption is negligible. RC function was assessed measuring absorption changes at 865 nm following excitation by a photographic flash (time duration 1 msec). The flash intensity was varied by neutral density filters and, to achieve energies above 1 mJ/pulse by increasing the flash duration time.

Experimental Results

LHCP in phospholipid vesicles

Fig. 1 shows a typical fluorescence spectrum of LHCP reconstituted into DMPC vesicles for a temperature above (solid line) and below (dotted line) the gel/fluid phase transition ($T_c = 23$ °C) and for two different excitation wavelengths. Increasing the temperature does not cause, as usually observed, a decrease but an increase in intensity of the dominant dimeric emission at 860 nm. On the other hand the shape of the spectrum is basically preserved. This is especially noticable considering the band near 800 nm which usually increases on protein degradation [7]. The weakness of this band corresponding to

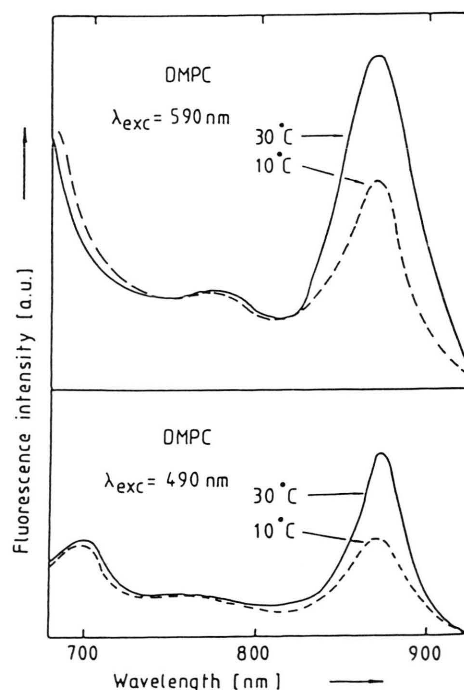


Fig. 1. Fluorescence spectrum of LHCP in DMPC vesicles for two different excitation wavelengths λ_{exc} (indicated) and for a temperature above (30 °C) and below (10 °C) the gel/fluid phase transition. Protein/lipid ratio 1:5200, lipid concentration 10^{-3} M.

monomeric BChl shows that energy transfer from BChl to the "dimeric" trap which absorbs near 850 nm is very effective. This trap is called dimeric, because it probably corresponds to two excitonically coupled BChl. It also indicates only little BChl that might be lost from the protein during reconstitution is dissolved within the membrane. In addition the emission of a photoproduct near 700 nm is almost independent of temperature. The observed changes are independent of excitation intensity, of excitation wavelength (varied between 450 nm and 650 nm) and of LHCP/lipid ratio (varied between 1:7000 and 1:1500). The 700 nm photoproduct is not discussed furtheron, as it does not affect fluorescence quenching and is not relevant considering the results below.

A comparison of the temperature dependence of fluorescence intensity (at 860 nm and light scattering is given in Fig. 2 to 4. The transmission signal clearly shows onset, center and end of the phase transition in accordance with literature data [11]. Obviously there is a correlation between phase transition and fluorescence change and this contains one main message of this work.

Considering the case of LHCP in DPPC (Fig. 2a) one realizes that fluidization of the membrane causes a fluorescence increase by about 60%, but this change occurs by about 3 °C below the transition temperature.

Qualitatively the same holds for LHCP in DMPC (Fig. 2b), the shift of the "fluorescence detected" transition is only 1 °C but occurs into the same direction.

The change is reversible with only a slight hysteresis comparing heating and cooling runs.

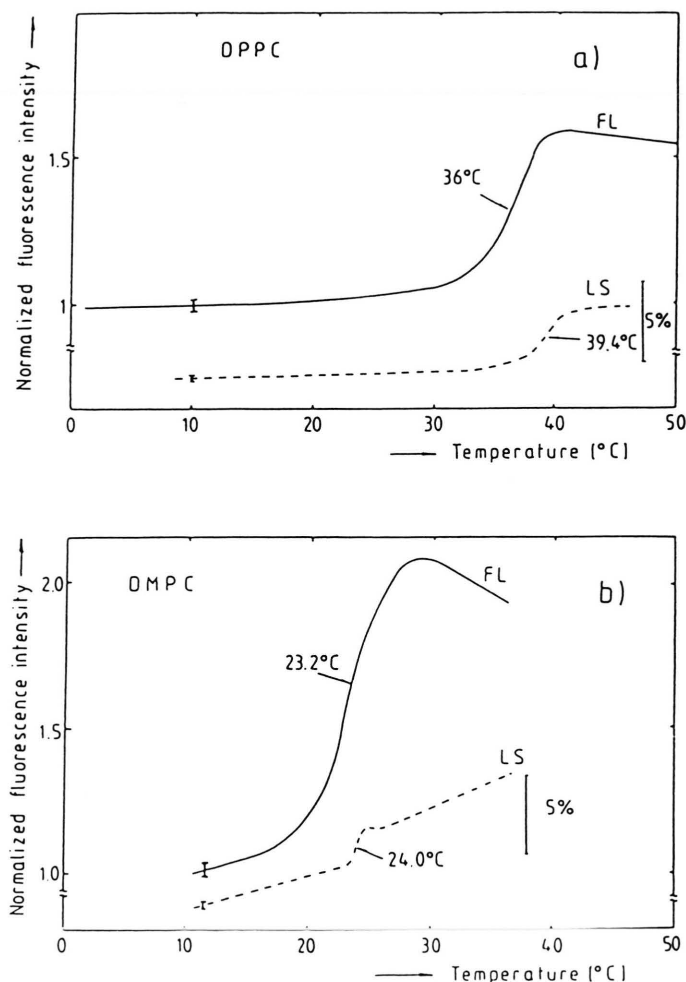


Fig. 2. Fluorescence intensity (FL) normalized to the value at 10 °C and transmission change due to light scattering (LS) as a function of temperature for LHCP in DPPC vesicles (Fig. 2a) and in DMPC vesicles (Fig. 2b). Protein/lipid ratio 1:4300 (Fig. 2a) and 1:3000 (Fig. 2b), respectively. Lipid concentration 10^{-3} M. The bar indicates 5% transmission change.

In accordance with the findings of Fig. 2a, b one observes no discontinuity in the fluorescence intensity versus temperature plot for LHCP in DLPC because the phase transition, expected below 10 °C does not appear in the temperature range studied by us.

Preparing lipid mixtures one might assume that the protein selectively accumulates one of the lipid components in its environment to reduce mismatch in the hydrophobic region. This should result in temperature induced changes of this component modulating the fluorescence. A mixture of DLPC and DPPC is expected to phase separate at any temperature due to the large difference in hydrocarbon chain length [12]. This should result in an extremely broadened transition and in favourable cases two transitions may show up [11]. We indeed observe a broad transition by light scattering but no concomittant fluorescence change for a 1:1 mixture (Fig. 3a). This indicates that the protein is embedded exclusively in the fluid lipid which is the DLPC enriched phase below 40 °C. For a 3:1 mixture of the two immiscible lipids DMPC and DSPC, respectively, light scattering basically shows only the broadened DMPC transition shifted by about 2 °C. Again, changes in fluorescence yield appear at a 2.5 °C lower temperature (Fig. 3b). The temperature of largest slope is identical to that measured with pure DMPC. A less steep component in the fluorescence transition appears additionally at temperatures between 26 °C and 32 °C.

For an alloy of the miscible lipids DMPC and DPPC we also observe a broadened transition at a temperature between the transition temperatures of the pure components and the corresponding fluorescence curve is shifted by 3 °C up compared to pure DMPC and by 5 °C down compared to the phase transition of the mixture.

The fluorescence decrease accompanying solidification of the lipid environment is not specific for molecules with the choline head group but observed also for a charged lipid like DLPA (Fig. 4a). In that case one can shift the transition *e.g.* by screening head group repulsion by divalent ions [13]. This also leads to a corresponding shift in the fluorescence curve. The original situation can be restored by complexing the divalent ion by EDTA. This is demonstrated considering the fluorescence changes at 860 nm measured in the experiment of Fig. 4b. We confirmed that the fluorescence decrease at 860 nm

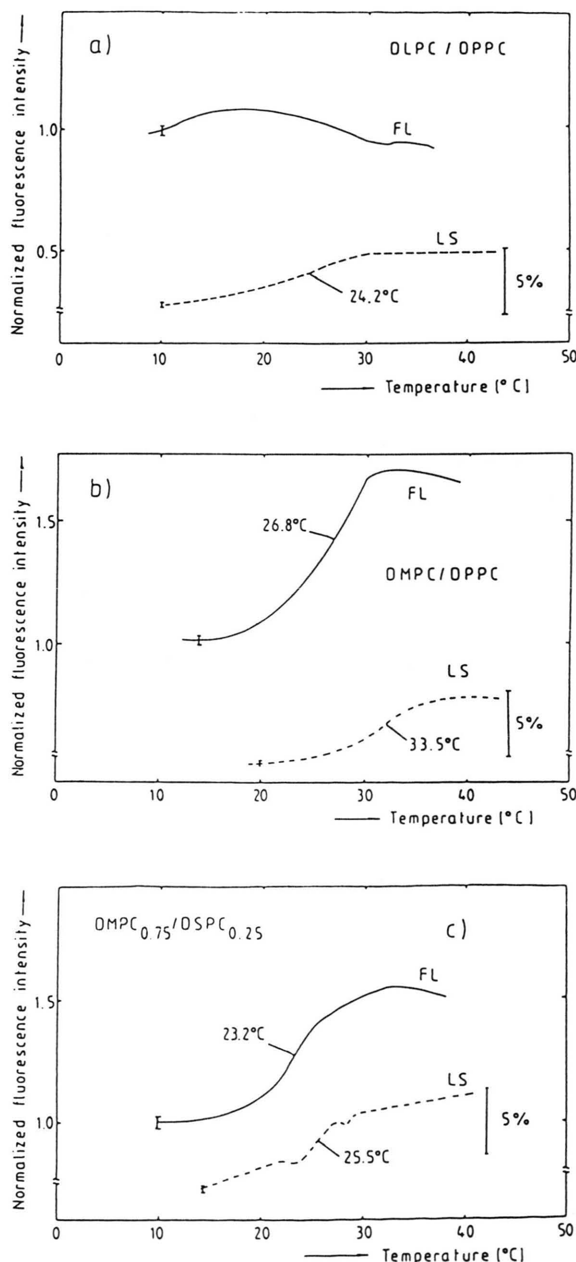


Fig. 3. Fluorescence intensity (FL) normalized to the value at 10 °C and transmission change due to light scattering (LS) as a function of temperature for LHCP in lipid mixtures. Fig. 3a: DLPC/DPPC (1:1); Fig. 3b: DMPC/DSPC (3:1); Fig. 3c: DMPC/DPPC (1:1). Protein/lipid ratio 1:2000 (Fig. 3a), 1:1800 (Fig. 3b) and 1:3600 (Fig. 3c). Lipid concentration 10^{-3} M. The bar indicates 5% transmission change.

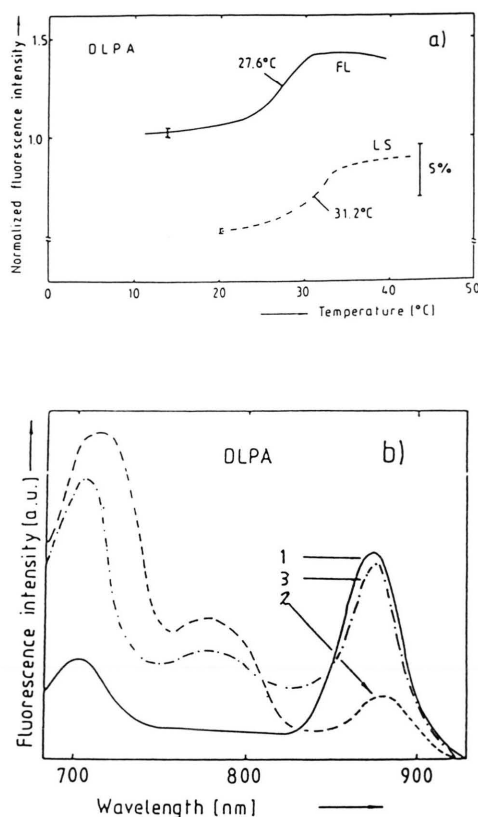


Fig. 4. a. Fluorescence intensity (FL) normalized to the value at 10 °C and transmission change due to light scattering (LS) as a function of temperature for LHCP in DLPA vesicles. Protein/lipid ratio 1:6800, 10 mM Tris buffer, 30 mM NaCl, 5 mM EDTA and 10^{-3} M lipid concentration. b. Fluorescence spectrum of LHCP in DLPA vesicles before (——, curve 1) and after (---, curve 2) incubation of 7 mM $MgCl_2$ and after further adding of 10 mM EDTA (-·-·-, curve 3). Starting conditions as in Fig. 4a. $T = 20$ °C.

in spectrum 2 is due to Mg^{2+} binding to the membrane in a reference experiment with LHCP in DLPC that does not bind Mg^{2+}. In that case one indeed does not observe cation induced fluorescence changes (for Mg^{2+}-concentrations below 5 mM). One also realizes the strong increase of the band at 700 nm corresponding to a photoproduct. Obviously during this experiment a fraction of BChl within the proteins is damaged making a more quantitative analysis difficult.

LHCP and RC in phospholipid vesicles

The above experiment showed that the membrane environment affects one functional parameter of LHCP, the fluorescence yield. The prime natural function, however, is to transfer absorbed light energy to the RC. This function can be studied by reconstitution of LHCP together with RC into lipid vesicles and measuring the LHCP fluorescence quenching and the electron transfer within the RC after LHCP excitation.

Fig. 5 shows the change in the LHCP fluorescence spectrum in presence and absence of RC. One observes a drastic reduction in intensity on RC reconstitution. To assure that this is due to energy transfer and not to any preparation artefacts also the RC free sample was subjected to the procedure applied to additionally reconstitute the RC. Thus the two samples studied in Fig. 5 present the same preparation history. An additional proof that fluorescence quenching is due to energy transfer although the protein concentrations are rather small, results from the dependence of LHCP fluorescence on LHCP/RC ratio (Fig. 6). One clearly observes a fluorescence increase with increasing LHCP/RC ratio after LHCP excitation. This increase is almost linearly up to ratios near two and then seems to occur steeper. However, the data scatter is too large, probably due to heterogeneous protein concentrations on different vesicles, to allow determinations of binding constants or binding stoichiometry. In fact freeze-etch-electron micrographs showed that RC are statistically distributed within fluid phase vesicles, but RC concentrations vary considerably [6]. LHCP could not be visualized by this technique. Having shown that fluores-

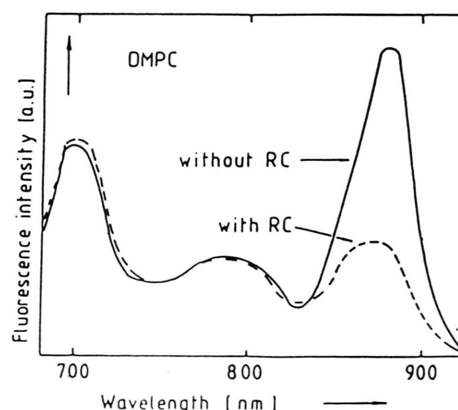


Fig. 5. Fluorescence spectrum of LHCP in DMPC vesicles without (——) and with (– – –) additionally reconstituted RC to achieve a RC/lipid ratio of 1:3800. LHCP/lipid ratio 1:5900 and lipid concentration 10^{-3} M. T = 18 °C.

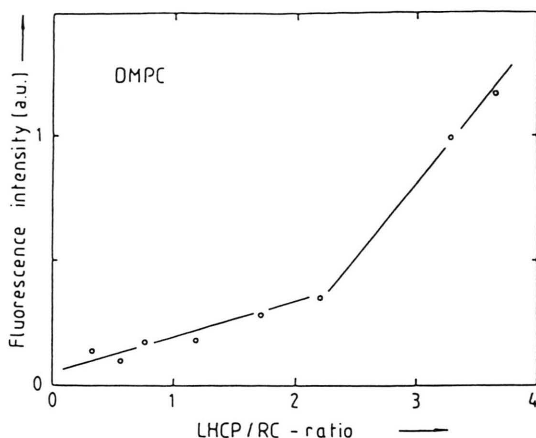
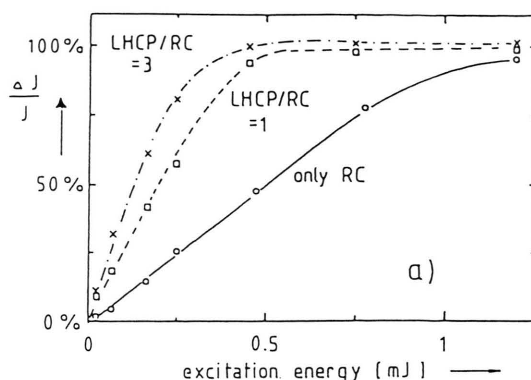


Fig. 6. Fluorescence intensity as a function of LHCP/RC ratio for a fixed RC/lipid ratio of 1:7500. 10^{-3} M DMPC, T = 20 °C, excitation wavelength 590 nm.



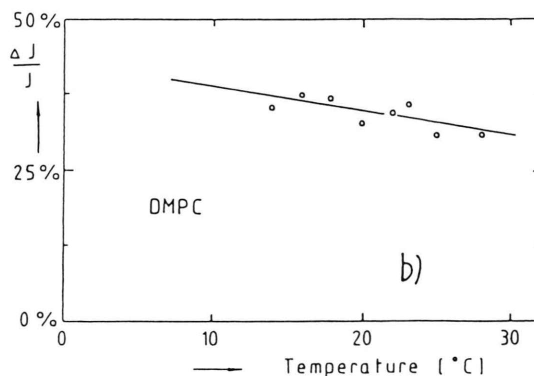


Fig. 7. a. RC absorption change ΔI (at 860 nm) normalized to the absorption value at complete bleaching, I, as a function of excitation energy per pulse. Excitation wavelength was selected between 430 nm and 490 nm to predominantly excite the LHCP carotenoid absorption bands. RC/lipid ratio 1:2800, LHCP/RC ratios indicated in the figure. 10^{-3} M DPPC, T = 25 °C.
b. $\Delta I/I$ as a function of temperature for a pulse energy of 0.1 mJ, a LHCP/RC ratio of 1.5, a RC/lipid ratio of 1:2400 and 10^{-3} M DMPC.

cence quenching is due to energy transfer one can study the influence of the phase state comparing the temperature dependence of fluorescence intensity in presence and absence of RC. One indeed observes a fluorescence reduction by a ratio which is independent of temperature. This indicates that energy transfer is independent of phase state.

Complementary information on energy transfer is expected from a measurement of the RC absorption change (at 860 nm) after LHCP excitation. Fig. 7a gives a measurement of the RC bleaching as a function of excitation intensity for various LHCP/RC ratios. The absorption change is virtually linear in excitation intensity for low intensities and saturates for high light levels (not shown). Saturation is reached earlier for high LHCP ratios because in that case the number of absorbed photons is larger. To conclude on energy transfer LHCP/RC ratio and excitation intensity were chosen to be near the linear ranges at low excitation intensities of Fig. 7a. The temperature dependence of absorption change given in Fig. 7b then again shows that energy transfer hardly depends on temperature.

Discussion

Structural change of protein or its aggregation state

As we have shown above the gel/fluid phase transition is accompanied by light scattering changes, we have to prove that the observed fluorescence changes are due to a structural change involving the protein and not due to light scattering.

- The strongest argument excluding the influence of light scattering results from the fact that scattering and fluorescence changes do not occur at the same temperature.
- In addition we quantitatively assessed this interdependence by measuring the transmission change of the sample during fluorescence measurements. The turbidity changes due to lipid phase transition are below 10% and thus cannot explain fluorescence changes which amount up to 100%.
- The transmission changes hardly influence the

fluorescence intensities which can also be deduced from the temperature invariant emission of a photoproduct at 700 nm (Fig. 1).

Hence, having demonstrated the existence of a structural change we may ask for the mechanism leading to the fluorescence decrease. One reason might be that the intermolecular energy transfer to the fluorescing dimeric trap is hindered. Arguments against this are that changes are identical irrespective of excitation into the carotenoid of BChl absorption band. If energy transfer from monomeric to dimeric BChl would be reduced this would show up in an additional fluorescence emission at 800 nm in contrast to the observation.

It is also impossible that nonlinear processes like singlet-singlet-annihilation [14] that might be affected by a phase transition are responsible for the intensity change. These would depend on excitation intensity, in contrast to our findings. Although we have no indication on it, we can, however, not exclude energy transfer to a trap, a dimer, aggregate or impurity.

Yet it is more probable that a radiationless transition is becoming more effective due to stronger coupling in the state at low temperatures. This has been discussed recently by Pearlstein [15] and also explains the strong dependence of LHCP fluorescence quantum yield on surface pressure in monolayers [7] and on type of detergens in micelles [16].

Concluding this section we should stress that the structural changes are reversible and do not involve a protein degradation.

At present we cannot discriminate between a structural change inside the monomeric protein unit and one where the aggregation state changes. The latter would in fact be indicated in accordance with the model in ref. [15], where a change in symmetry of the hexameric or dodecameric protein aggregates would shift the positions of the excitonic sublevels.

Membrane influence on structural change

We have shown above by fluorescence measurements that a lipid phase transition affects protein structure. This trivially proves that LHCP is embedded in the membrane but, more importantly, points to the strong influence of membrane elasticity on protein function. There will always be a mismatch between protein and membrane structure leading to elastic stress at the protein/lipid boundary (Fig. 8) [17]. The stress is drastically increased on going to the gel state due to increase in elastic modulus enforcing a protein structure that may differ from that in a more fluid environment.

On the other hand during the phase transition the membrane structure, in special thickness [18, 19] and hydrocarbon chain order [20] are varied. We have shown recently [6] that the RC may shift lipid phase transition temperatures in both directions depending on the relative thickness of hydrophobic parts of protein (d_p) and membrane (d). This shift is not observed for LHCP probably due to the too small membrane area ratio (<1%) occupied by the protein at the concentrations applied by us. Yet the ideas then advanced based on a mattress model [6] may also be applicable in the present situation:

If d_p is smaller than the mean thickness d of the hydrophobic part of the membrane in gel and fluid state the protein favours the fluid phase in its environment. This surely holds for LHCP in membranes of lipids containing hydrocarbon chains of lengths

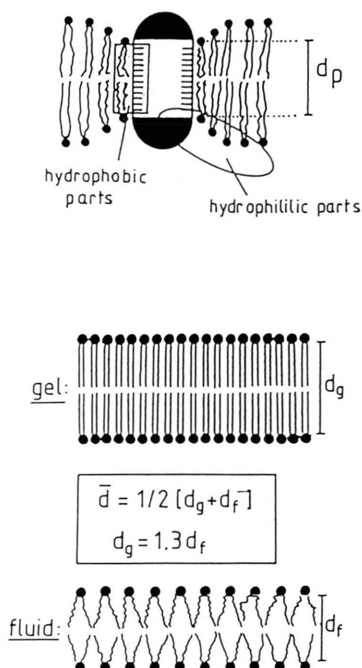


Fig. 8. Sketch of elastic deformations caused by protein incorporation and definition of parameters characterizing the membrane thickness. d = mean lipid chain length; d_f and d_g = membrane thickness in the fluid and gel state; d_p = length of the hydrophobic part of the protein.

larger than or equal to 16 C-atoms, because d_p is reported as 30 Å [21] and d for DPPC (16 CH_2 groups) is reported as 30 Å [22].

Hence we expect that, increasing the temperature from below, the protein environment starts to melt at a lower temperature than the undistorted membrane (Fig. 8). This requires that the protein acts as a defect in the gel phase membrane where melting of the whole membrane starts or that it melts at a separated temperature. The results in Fig. 2–4 show that the latter holds true: The fluorescence begins to increase on increasing the temperature at a point up to 5K lower than the one corresponding to the onset of the phase transition.

The shift is especially well pronounced for the DMPC/DPPC mixture. There the phase transition is broadened and the "fluorescence transition" sets in almost at the same temperature as for pure DMPC bilayers. The change is terminated at a temperature of 31 °C, well below the value of 35 °C reported by Schmidt and Knoll [12] for the phase transition of the 1:1 mixture. This indicates a chain selective protein/lipid binding where the shorter of the two lipids favours the (short) protein. This interesting behaviour will be studied in future in further detail.

The finding on LHCP in DLPA demonstrated two facts:

(i) The shift between LHCP structural change and phase transition is not related to the P_{β}' ripple phase as DLPA does not exhibit this phase.

(ii) The influence on the protein via the membrane environment need not only be exerted by temperature but also by varying the chemical environment. This would be the more probable case in natural systems [23].

In addition it has been shown in monolayer experiments that LHCP tends to order DLPA by electrostatic binding [7]. The additional elastic force in a bilayer obviously overcompensates this leading to higher membrane disorder in the protein environment.

Protein/protein interactions

Since up to now it has not been possible to reconstitute LHCP and RC simultaneously into an artificial membrane system we have to prove that this has been successful in this work:

- Reconstitution of RC alone has previously been proven from electron microscopic data and from the influence on the lipid phase transition [6], reconstitution of LHCP alone is proven from the lipid influence on LHCP fluorescence. In both cases the successful reconstitution could also be assessed by spectroscopic analysis of the sediment obtained after centrifugation of the protein/lipid mixture.
- Reconstituting both proteins simultaneously we detected both proteins in the sediment and also determined the concentration of both by absorption spectroscopy. That both proteins are in fact in the membrane was confirmed by electron microscopy for RC and by the lipid influence on the LHCP fluorescence.
- Another strong argument in favour of a successful reconstitution of both proteins is the observation of highly efficient energy transfer discussed below. The latter would be not expected for a homogeneous RC distribution ($3*10^{-7}$M for conditions of Fig. 5) in a threedimensional solution.

Due to the existence of elastic forces one might assume that

a) dissimilar proteins tend to repel one another or
b) proteins are locally enriched in parts of the membrane, especially in the gel phase.

Energy transfer LHCP→RC in micelles is reported to be very efficient [24], and this process would be even further increased if the proteins patch within the membrane. The latter would hold especially on reducing the temperature but this is not observed in our experiments. Even the reduction in fluorescence quantum yield Φ_F is not linked with a reduced energy transfer due to a shortened fluorescence lifetime.

In a simple reaction scheme Φ_F and the quantum yield for energy transfer Φ_{ET} are related to the fluorescence rate constant k_F, the rate constant of radiationless transitions k' and the energy transfer rate k_{ET} according to:

$$\Phi_F = \frac{k_F}{k_F + k' + k_{ET}} \quad (1a), \quad \Phi_{ET} = \frac{k_{ET}}{k_F + k' + k_{ET}}. \quad (1b)$$

Reduced fluorescence in absence of energy transfer ($k_{ET} = 0$) requires an increased k'. This in turn would cause a reduced Φ_{ET} if the denominator in Eqn. (lb) would not be governed by k_{ET}. The latter would require $\Phi_{ET} \approx 1$, *i.e.* a saturation of energy transfer rate with acceptor concentration and $\Phi_f \approx 0$. This was not the case. Therefore the temperature

invariant Φ_{ET} has to be explained by a model assuming a heterogeneous protein distribution on the membrane surface: One fraction of LHCP is linked to the RC thus showing no fluorescence, the other one is separated from it at a large distance with $k_{ET}=0$. Besides explaining the temperature dependence of Φ_{ET} the model accounts for the following observations:

- The fluorescence increase with increasing LHCP and decreasing RC concentrations is due to different relative amount of the two LHCP fractions.
- RC concentration variations on different vesicles were observed electron microscopically. Unfortunately we could not unequivocally observe LHCP in freeze-etch electron micrographs. This may be due either to their small size or to formation of heterogeneous aggregates that cannot be distinguished from artefacts.
- The energy transfer was observed to be highly efficient at RC/lipid ratios as small as 1:4000 corresponding to a density of $\varrho = 10^{11}/\text{cm}^2$. This density can be transformed into a typical energy transfer radius R_{ET} according to

$$R_{ET} = \sqrt{1/(\pi\varrho)} = 18\ \text{nm}. \qquad (2)$$

This value derived from the assumption of a statistical RC distribution is too large compared to a typical Förster radius (≈ 50 Å), indicating an inhomogeneous protein arrangement.

The fraction with large k_{ET} may be understood on one hand as one with tightly bound LHCP and RC, as we have not been able to reduce its fraction *via* changes in phase state and type of lipids. But this need not necessarily be a chemical bond as we would not be able to distinguish between that and a structure where the RC is physically entrapped within a larger LHCP aggregate. On the other hand it may also be understood as a dynamic equilibrium between states of associated and unassociated proteins. In the latter case however it would be surprising that this equilibrium is temperature independent.

Acknowledgements

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