# The role of chromophore in the lipid–protein interactions in bacteriorhodopsin–phosphatidylcholine vesicles

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**Abstract By fluorescence and phase properties of a 1-acyl-2-[8-(2-anthroyl)-octanoyl]-*sn*-glycero-3-phosphocholine probe, the influence of the chromophore on the phase transition of bacteriorhodopsin–lipid vesicles was investigated. It was observed that removal of the chromophore led to the down-shifting of the phase transition temperatures. The temperatures corresponding to the beginning and ending of the gel–liquid phase transition were also influenced. This demonstrated that the liquid phase is reached more easily when the chromophore is bleached. The results indicate that removal of the chromophore alters the protein–lipid interactions. It is suggested that this alteration might be related to the change in the lipid molecular packing.** 

*Key words:* Lipid–protein interaction; Chromophore; Bacteriorhodopsin; Lipid phase transition; Fluorescence excitation spectrum

## 1. Introduction

Bacteriorhodopsin (bR), the unique protein present in the purple membrane (PM) of *Halobacterium salinarum*, transports protons from the interior to the exterior of the cell [1]. The driving force for this transport is provided by light, which is collected by a retinal molecule bound to the protein by a protonated Schiff base. Besides acting as an antenna to capture photon energy and to transmit this energy to the protein moiety, retinal also influences the structure of bR [2,3]. After bleaching the retinal, the PM did not show the characteristic crystalline arrangement of the protein in the lipid bilayer [4–6]. It was demonstrated that retinal removal has an effect on both protein tertiary structure and the loops [7]. Particularly, the experiments argue in favour of a more open conformation of the loops in the apomembrane, and suggest that the retinal molecule is able to elicit detectable changes in the external loops by altering the compactness of the transmembrane helices [8–10]. A more open conformation of the protein structure on bleaching has also been suggested on the basis of spectroscopic studies [11,12].

The structure of bR is affected by retinal [13–15]. bR has a strong influence on the phase transition of the various phosphatidylcholine species in which it was reconstituted [16,17]. Therefore, one may expect that retinal should influence the bR–lipid interaction. Indeed, there are several hints in the literature that the removal of the chromophore led to small alterations in the phase properties of the lipids [10,17–19]. But they were considered too small to indicate statistically significant differences and it was stated that removal of retinal did not alter the protein–lipid interactions [17,18]. One of the reasons for the failure to find reliable evidence may be related to the fact that the addition of bR to the lipids resulted in a broadening of the phase transition [8,18]. Therefore, this could mask more complex phase behaviours due to the presence of retinal.

These facts prompted us to investigate the influence of the retinal molecule on the phase transition of a bR–lipids system. We used the approach developed by Piknova et al. [16], which uses the fluorescence and phase properties of the 1-acyl-2-[8-(2-anthroyl)-octanoyl]-*sn*-glycero-3-phosphocholine (Ant) probe. Ant enables gel–liquid phase transitions of lipids to be detected even if the underlying chain melting process is poorly co-operative. This is a marked advantage over other techniques, which need enough co-operativity for a phase transition to be clearly detected and analysed [20–24]. This technique allows the determination of not only the temperature of main phase transition but the temperature of pretransition as well. Moreover, the temperatures corresponding to the beginning and ending of the gel–liquid phase transition can be determined. Therefore, the more complex phase behaviours of bR–lipids and bacterioopsin (bO)–lipids could be observed.

## 2. Materials and methods

Ant was synthesised according to a previously described method [25]. Dilauroylglycerophosphocholine (Lau), dimyristoylglycerophosphocholine (Myr) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma. All-*trans*-retinal was purchased from Aldrich. β-$C_8$-BODIPY 500/510 $C_5$-HPC (BDY) was obtained from Molecular Probes. Purple membranes were obtained from *Halobacterium salinarum*, strain S9, according to the standard procedure [26].

Reconstitution of bR into Lau or Myr vesicles was performed according to the methods described by Piknova et al. [16] (measurements with Ant) or Rehorek et al. [18] (measurements with DPH). bR was bleached following [17,18] for experiments with DPH or following [26,27] for experiments with Ant. Regeneration of bO–lipid vesicles with all-*trans*-retinal has been performed according to [27].

Absorption spectra were recorded with Shimadzu 2000. Fluorescence excitation ($F_{Exc}$) and emission ($F_{Em}$) spectra were recorded with a Perkin-Elmer spectrofluorimeter, LS 50, equipped with a home-made thermostated cuvette holder (temperature stabilisation,

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*Abbreviations:* BDY, β-$C_8$-BODIPY 500/510 $C_5$-HPC; bR, bacteriorhodopsin; bO, bacterioopsin; PM, purple membrane; Ant, 1-acyl-2-[8-(2-anthroyl)-octanoyl]-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Lau, dilauroylglycerophosphocholine; Myr, dimyristoylglycerophosphocholine

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±0.5°C). The wavelengths for fluorescence measurements were: $\lambda_{exc}$ = 340 nm, $\lambda_{em}$ = 460 nm for Ant and $\lambda_{exc}$ = 360 nm, $\lambda_{em}$ = 430 nm for DPH.

The steady-state fluorescence depolarisation measurements were performed on a spectrofluorimeter equipped with Glan–Thompson polarisers. Time-dependent fluorescence depolarisation measurements were carried out on a single-photon counting apparatus similar to that described by Rehorek et al. [17]. The pulse width was 1.0–1.3 ns full width at half maximum (FWHM). The lamp was operated at a pulse frequency of 30 or 50 kHz. The steady-state fluorescence anisotropy and time-dependent anisotropy were calculated according to standard methods [18].

## 3. Results and discussion

### 3.1. Phase behaviour of bR–phosphatidylcholine studied with DPH

The effect of the removal of the acceptor retinal on steady-state anisotropy of the donor DPH is demonstrated in Fig. 1. In the case of the phase transition curve for pure lipids, it gives only the temperature of the main gel–liquid phase transition at about 23°C. The addition of the protein to the lipids resulted only in a broadening of the phase transition, which remained centred around the main value for pure lipids. At all temperatures, the anisotropy for vesicles with acceptor (full symbols) is higher than that for the same samples after bleaching (open symbols). The donor order parameters calculated from time-dependent fluorescence depolarisation measurements for DPH in vesicles with higher bR concentrations were 0.622 and 0.522 for unbleached and bleached vesicles, respectively. The respective values for DPH in vesicles with lower bR concentration were 0.449 and 0.413. These values nicely match the values obtained by Rehorek et al. [17,18]. The results clearly demonstrate that the experiments with DPH are reproducible: the tendencies of the anisotropy changes and the values of order parameters obtained in the earlier experiments are held. In view of these facts, it is surprising that the authors disregarded the points for the highest bR/lipids ratio so as to justify the assumption that there is no statistically significant difference in order parameter in the bleached and unbleached states [17,18]. These results were interpreted as evidence that the removal of the retinal did not change the protein–lipid interactions.

The current results for DPH provide evidence that the earlier experiments performed with DPH should be reconsidered rather as a confirmation of the retinal influence on the protein–lipid interactions. This suggestion is further strengthened by experiments with Ant.

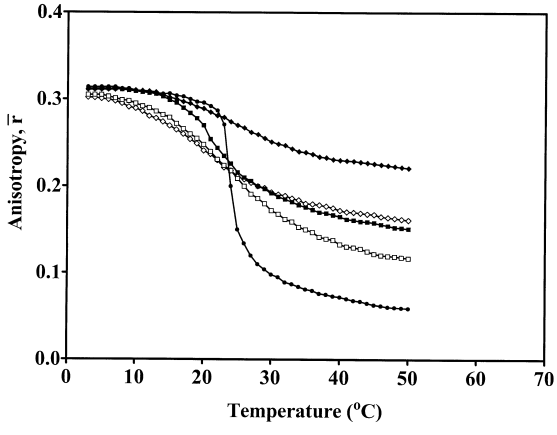


Fig. 1. Steady-state anisotropy ($\bar{r}$) of DPH as a function of temperature for unbleached and bleached bR–Myr vesicles at various bR/Myr molar ratios. bR/Myr ratio: 0, full circles; 0.01, squares; 0.02, diamonds. Full symbols, unbleached vesicles; open symbols, bleached vesicles.

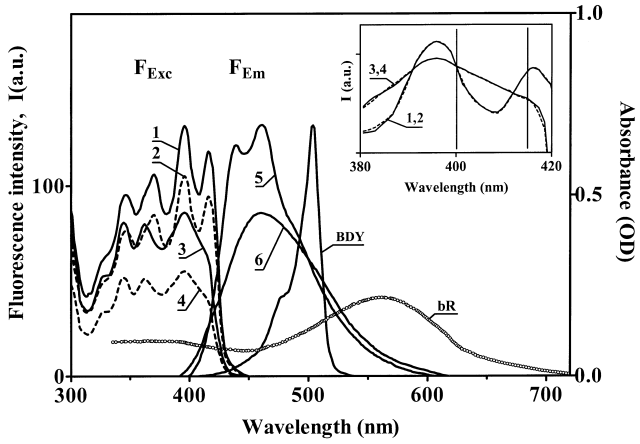


Fig. 2. Fluorescence excitation ($F_{Exc}$) and emission ($F_{Em}$) spectra of Ant in lipid vesicles. 0.5 mol/100 mol Ant (with respect to lipids) in bR/Myr 1:80 (mol/mol) recombinants, at temperatures of 10 (curves 1 and 5, respectively) and 30°C (curves 3 and 6, respectively). Dashed lines represent respective excitation spectra of Ant vesicles containing 1:200 mol/mol (labeled/unlabeled PC) BDY, at 10°C (curve 2) and at 30°C (curve 4). $\lambda_{exc}$ = 340 nm for the emission spectra, $\lambda_{em}$ = 460 nm for the excitation spectra. BDY, fluorescence excitation spectrum of BDY in Myr vesicles ($\lambda_{em}$ = 550 nm). bR, absorption spectrum of bR in Myr vesicles. The absorption spectrum refers to the right axes. Inset: Part of enlarged excitation spectra (curves 1–4) normalised to the value of fluorescence intensity at 400 nm. Vertical lines indicate the wavelengths of 415 and 400 nm for which $I_{415}/I_{400}$ was calculated.

### 3.2. The Ant probe

Fig. 2 shows the $F_{Exc}$) and $F_{Em}$ spectra of Ant in vesicles containing bR. The excitation and emission spectra of Ant can be used to monitor the phase transition of lipids [16]. Although for lipids in the fluid phase there is a loss of structure in the emission spectra (Fig. 2, curve 6), the excitation spectra remain structured and are a very suitable tool for studying subtle effects related to phase transition of the lipids ($F_{Exc}$ in Fig. 2, curves 1 and 3). The phase changes can be studied by following the spectral shift of the red-side band in the excitation spectra, which moves from 415 nm to 400 nm when lipids undergo a gel–liquid phase transition. This shift can be quantitatively assessed by measuring the ratio $I_{415}/I_{400}$ of the recorded fluorescence intensities [16,28]. This ratio takes values of 1.4–1.0 and 0.4 for lipids in the gel and liquid phase, respectively.

The approach with the registration of fluorescence excitation spectra of Ant for phase transition experiments has an excellent advantage over measurements with DPH. In the presence of energy transfer processes, the character of the fluorescence excitation spectra of Ant and, what is most important, the ratio $I_{415}/I_{400}$ of the diagnostic intensities are not changed (Fig. 2, curves 1–2, and 3–4, also inset). Hence, there is no need to make any corrections due to the energy transfer that may appear between Ant and the chromophore. This effect was examined in Ant/Myr vesicles into which acceptor molecules, BDY lipid probes, were incorporated. The presence of the acceptor (Fig. 2, BDY) caused a decrease of Ant fluo-

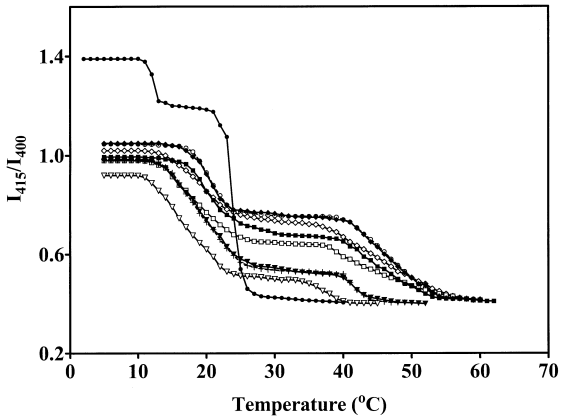


Fig. 3. Influence of the temperature on the phase state of bR–Myr recombinants with different molar ratios. bR/Myr molar ratios: 0, full circles; 0.002, triangles; 0.01, squares; 0.02, diamonds. Full symbols, unbleached vesicles; open symbols, bleached vesicles. Dashed lines with crosses and open circles represent phase transition for regenerated bR–lipid vesicles for 0.002 and 0.02 bR/Myr molar ratios, respectively.

rescence intensity due to the efficient energy transfer between the donor Ant and the acceptor (Fig. 2, dashed lines 2 and 4). However, the shape of the curves was not changed and the ratio $I_{415}/I_{400}$ calculated for curves without and with energy transfer remained the same (Fig. 2, inset).

### 3.3. Phase behaviour of bR–lipids and bO–lipids studied with Ant

Two lipid species, Myr and Lau, were used to prepare bR–lipid systems. It has already been demonstrated that particularly these two lipid species could facilitate the observation of bR effects on the phase behaviour of lipid vesicles [16–18].

*3.3.1. Phase behaviour of bR–Myr vesicles.* Fig. 3 (full symbols) presents the phase behaviour of bR–Myr vesicles at different bR/lipid ratios. The main gel–liquid phase transition of the pure Myr lipids was detected around 23°C (Fig. 3, full circles). The pre-transition for these lipids (around 13°C) was also detected. Similar values were obtained by Piknova et al. [16]. However, it should be emphasised that the pre-transition was not registered with other methods [8,18].

Addition of bR to Myr caused slight changes in the phase curves: the phase transition was triphasic with temperatures of phase transition around 20–23°C and 42–45°C. An increase of the bR concentration slightly changed the proportions of low-melting and high-melting phases with a very small upshift in the transition temperatures. In the gel phase smaller $I_{415}/I_{400}$ ratios were calculated in the presence than in the absence of bR suggesting some loosening of the lipid packing [16].

Fig. 3 (open triangles, squares and diamonds) clearly illustrates that the removal of the chromophore affects the phase curves. The chromophore bleaching caused that the $I_{415}/I_{400}$ ratios were even lower than those for unbleached samples, suggesting that the loosening of the lipid packing in the gel phase was strengthened. This is also confirmed by lowering the temperature of phase transition. The down-shift in temperatures corresponding to the beginning of the gel–liquid phase transition suggests that lipids achieve the liquid phase more easily when bleached bR is present. However, the triphasic character of the phase transition was not affected by retinal removal. This should be emphasised in view of the fact that simple incorporation of bR into Myr resulted in a triphasic phase transition. It may mean that there is a kind of hierarchy in bR effects on lipid phase transition: secondarily caused by active centre chromophore, and primarily induced by opsin.

*3.3.2. Phase behaviour of bR–Lau vesicles.* Fig. 4 (full symbols) presents the phase behaviour of bR–Lau vesicles at different bR/lipid ratios. The main gel–liquid phase transition of pure Lau was detected around −3°C which is in reasonable agreement with values between −6 and −1°C [16], and −2.0 and 0°C [29].

Addition of bR to Lau had a strong influence on the lipid phase transition: the higher the bR concentration, the higher the temperature of the lipid phase transition (Fig. 4, full symbols). The phase transition became triphasic at higher bR concentrations with temperatures of phase transition around 0–3°C and 36–39°C. The value $I_{415}/I_{400}$ progressively increases with increasing bR concentration, suggesting an increase in the molecular packing of the lipids in the gel phase. This molecular packing is weakened due to chromophore removal. The bleaching of vesicles resulted in a progressive down-shift in the phase transition temperature of the lipids (Fig. 4, open triangles, squares and diamonds). The temperatures corresponding to the beginning of the gel–liquid phase transition were again shifted towards lower values. The lack of chromophore in bR structure slightly facilitated the transition of protein–lipid system into the liquid phase.

The postulated minor effect of chromophore on lipid phase transition can be clearly confirmed by the change in character of phase transition dependent on the bR–Lau ratio. The phase transitions were biphasic at low bR–Lau ratios. Instead, an increase of bR content caused the phase transitions to become triphasic. The removal of chromophore did not essentially change this effect, confirming that the major interaction responsible for lipid phase transition is the interaction between opsin and lipids.

The recorded differences between lipid phase transition of unbleached and bleached vesicles were not dramatic, but could not be interpreted, as the bleaching does not alter the

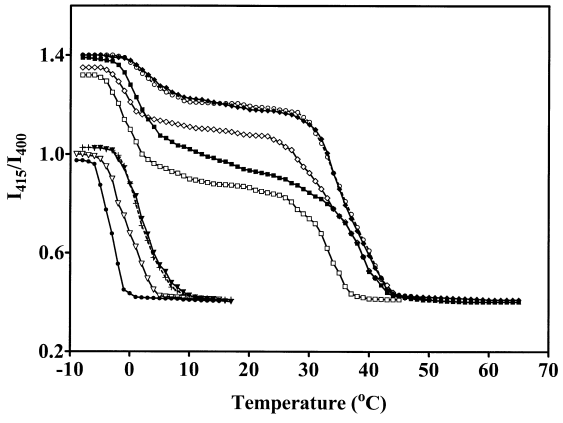


Fig. 4. Influence of the temperature on the phase state of bR–Lau recombinants with different molar ratios. bR/Lau molar ratios: 0, full circles; 0.002, triangles; 0.01, squares; 0.02, diamonds. Full symbols, unbleached vesicles; open symbols, bleached vesicles. Dashed lines with crosses and open circles represent phase transition for regenerated bR–lipid vesicles for 0.002 and 0.02 bR/Lau molar ratios, respectively.

protein–lipid interactions [17,18]. The presented results demonstrate the differences in phase behaviour between unbleached and bleached vesicles. The shift of the phase transition temperatures and the shift of the temperature corresponding to the beginning of the gel–liquid phase transition suggest that removal of the chromophore affects the protein–lipid interaction which can be manifested by a slight loosening of the molecular packing of the lipids.

This may also be reflected by a decrease in the value of order parameter determined with DPH. Hence, based on the above-presented results, it should be stated that results presented in [17,18] were slightly misinterpreted: the two last points from the discussed papers should not be discarded and their results should be reconsidered rather as a confirmation of the influence of retinal on the protein–lipid interactions.

*3.3.3. Phase behaviour of regenerated bR–lipid vesicles.* The chromophore affects the opsin structure. Therefore, it was suggested that retinal bleaching might alter the protein structure with respect to the arrangement of the amino acids in the chromophore binding site [30]. Hence, the changes observed in the phase transition may not be caused by a chromophore absence, but rather by the procedure of the chromophore removal from native bR.

The experiments with bR regenerated from bO and retinal helped to distinguish between these two effects. Figs. 3 and 4 present a comparison of the previously discussed results with phase transition for regenerated bR–lipid vesicles (dashed lines with crosses and open circles). The results are presented for low (0.002) and high (0.02) molar bR–lipid ratios. However, the phase curves for regenerated bR–lipid systems do not deviate markedly from those of native bR. These results support the conclusion that the absence of chromophore in the protein structure was the reason for the changes observed in phase transitions.

It is tempting to draw the more general conclusion that the resultant PM structure (and its function) defined by protein, specific lipids, and protein–lipid interactions is determined by the whole bR molecule: the protein plus the chromophore and the interaction between them. The specific feedback between the chromophore and the protein determines the entire bR action as a biological unit.

The question, however, to what extent retinal influences protein–lipid interactions remains unanswered. Studies into specific retinal analogues should help to answer this question.

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