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ARTICLE

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The role of retinal in the long-range protein-lipid interactions in bacteriorhodopsin-phosphatidylcholine vesicles

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Abstract The effects of bacteriorhodopsin analogues and the analogues of a bacteriorhodopsin mutant (D96N) on the lateral organization of lipids have been investigated with lipid species with a variety of acyl chain lengths. The analogues, obtained by regeneration of bacterioopsin or mutant opsin with 14-, 12-, 10-, or 8-fluororetinal, were reconstituted with 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine, and 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine. The phase behavior of the protein-lipid systems was investigated at different temperatures and different protein/lipid molar ratios by analyzing the fluorescence and phase properties of the 1-acyl-2-[8-(2-anthroyl)octanol]-*sn*-glycero-3-phosphocholine probe. The (8,10,12)-bacteriorhodopsins had a similar effect on the lipid phase transition to that induced by native bacteriorhodopsin: a rigidifying effect on the three shorter lipid species and a fluidifying effect on the longest-chain lipids used. The substitution of retinal with 14-fluororetinal resulted in much stronger effects of the protein on the lipids: a more pronounced up-shift of the lipid phase transition temperature, a rigidifying effect on all the lipids used, and an elongation of the distance over which the hydrophobic thickness of the lipid bilayer was perturbed by the protein. Evidence was provided that retinal contributed to the long-range protein-lipid interactions in bacteriorhodopsin-phosphatidylcholine vesicles. The extent of this contribution was dependent on the retinal structure in close vicinity to the Shiff base and on the compactness of the protein structure.

Key words Retinal · Bacteriorhodopsin · Lipids · Phase transition · Excitation spectra

Abbreviations *(14,12,10,8)-F-bR*: bacteriorhodopsin with retinal fluorinated at the 14-, 12- ,10-, or 8-position · *(14,12,10,8)-F-D96N*: bacteriorhodopsin mutant with retinal fluorinated at the 14-, 12-, 10-, or 8-position · *Ant*: 1-acyl-2-[8-(2-anthroyl)octanol]-*sn*-glycero-3-phosphocholine · *bO*: bacterioopsin · *bR*: bacteriorhodopsin · *Lau*: 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine · *Myr*: 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine · *Pam*: 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine · *PM*: purple membrane · *Ste*: 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine

Introduction

Integral membrane proteins are crucial to a wide variety of cellular activities (Tanner 1987; Boyd et al. 1998). However, the exact mechanisms by which protein behavior is modulated by the surrounding lipid matrix are unclear. Studies on the effect of lipids on membrane proteins have focused primarily on changes in the structure and function of the proteins (Froud et al. 1986; Riegler and Mohwald 1986; Barnett et al. 1996). In some cases, lipids may affect the relative populations of the active and inactive conformations of the membrane proteins and thereby affect the efficiency of their action (Brown 1994; McCallum and Epand 1995).

However, when proteins are incorporated in lipid bilayers, the structural and dynamic properties of the lipids are usually changed (Sperotto and Mouritsen 1991, 1993). Therefore this may have an effect on the efficiency of a whole biological segment responsible for the regulation of membrane-localized biochemical reactions (Melo et al. 1992; Thomson et al. 1995). For example, models of localized energy coupling by proton

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gradients require a transient confinement of protons near the membrane, allowing lateral proton transport along the surface between the proton source and sinks (Ferguson 1985). Hence, the resultant ATP production will be affected not only by the efficiency of the proton source but by long-range proton diffusion along the surface of the membrane as well.

Several important questions related to the perturbation of lipids induced by incorporated proteins remain unanswered (Rehorek et al. 1985; Riegler and Mohwald 1986; Stelzer and Gordon 1986; Dumas et al. 1997; Schram and Thompson 1997). Is the perturbation caused by the proteins restricted to a small neighborhood around them (boundary lipids), or do these changes have a long-range character and extend to bulk lipids through non-specific (or specific) protein-lipid interactions? How are the perturbations induced: by the whole protein or by parts of the protein, and which play a major role in effective protein-lipid interaction? What is the role (if any) of the reactive center of the protein complex in the protein-lipid interactions?

These questions are particularly important for bacteriorhodopsin (bR), the unique protein present in the purple membrane (PM) of *Halobacterium salinarum*, which transports protons from the interior to the exterior of the cell (Lanyi 1993). 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. After bleaching, the membrane does not show the characteristic crystalline arrangement of the protein in the lipid bilayer (Stoeckenius and Bogomolni 1982). Furthermore, bacterioopsin (bO) incorporated into liposomes becomes leaky to protons, whereas bR is impermeable (Burghaus and Dencher 1989). Retinal removal affects the properties of the cation-binding sites (Dunach et al. 1989). The chromophore of bR has an effect on both the protein tertiary structure and the loops (Cladera et al. 1992). Particularly, deuterium exchange experiments argue in favor of a more open conformation of the loops in the apomembrane, and suggests that the retinal molecule is able to elicit detectable changes in the external loops by altering the compactness of the transmembrane helices (Cladera et al. 1996). A more open conformation of the protein structure on bleaching has been also suggested on the basis of spectroscopic studies (Becher and Cassim 1977; Acuna et al. 1984).

bR has a strong influence on the phase transition of the various phosphatidylcholine species in which it is reconstituted (Dencher et al.1983; Piknova et al. 1993). On the other hand, as was already mentioned, the structure of bR is strongly affected by retinal. Therefore one can expect that retinal should influence the bR-lipid interactions. There are several hints that removal of the chromophore leads to small changes in the lipid phase transition curves and parameters (like fluorescence anisotropy, order parameters) describing the lipid's behavior with respect to the unbleached vesicles (Heyn et al. 1981a; Rehorek et al. 1985). However, these were considered too small to indicate the statistically significant differences and it was stated that retinal removal does not alter the protein-lipid interactions. One reason for the failure in finding reasonable evidence may be related to the methods used. Previous studies carried out with differential scanning calorimetry (Cladera et al. 1992) or fluorescence depolarization (Heyn et al. 1981a; Rehorek et al. 1985) revealed some alterations in the phase transition of the lipids. However, in all cases, the addition of the protein to the lipids resulted only in a large broadening of the phase transition, which remained centered around its nominal value. Therefore, this could mask more complex lipid phase behavior induced by retinal.

The approach developed by Piknova et al. 1993, seemed to us to be very useful. It uses the fluorescence and phase properties of the 1-acyl-2-[8-(2-anthroyl)octanol]-*sn*-glycero-3-phosphocholine (Ant) probe, which can detect gel/liquid phase transitions of lipids even if the underlying chain melting process is poorly cooperative. This is a marked advantage over other techniques, which need enough cooperativity for a phase transition to be clearly detected and analyzed. This technique allows determination not only of the temperature of the main phase transition but the temperature of the pretransition as well. Moreover, temperatures corresponding to the beginning and ending of the gel/liquid phase transition can be determined. Therefore, more complex phase behavior of bR/lipids can be observed.

These results prompted us to investigate the influence of the retinal molecule on the phase transition of the bR/lipid system. We have used retinal analogues. The incorporation of retinal analogues with a changed shape or altered electronic properties into the binding site of bR has been used to strengthen or to diminish the feedback between retinal and the protein surroundings (Govindjee et al. 1994; Kollbach et al. 1998). We have applied fluorinated retinal analogues. Since the fluorine atom is highly electronegative, its substitution for a hydrogen atom in the retinylidene chromophore can affect not only the electronic properties of the chromophore but also the interaction of the chromophore with opsin, especially the polarized or charged amino acid residues near the chromophore. The fluorine retinal analogues were (and are) widely used in studying different retinal proteins like rhodopsin (Shichida et al. 1987), retinochrome (Sekiya et al. 1992; Sekiya and Yoshihara 1995), and bR (Tierno et al. 1990; Kollbach et al. 1998).

We have also used a D96N mutant (Asp96 of wild-type bR is replaced with Asn). It was postulated that this site-specific mutation should affect the protein conformation (Brown et al. 1997) and may disturb the tight packing of bR (Kikukawa et al. 1995, 1997), and therefore the retinal influence on the rigid membrane lipids might be potentially enhanced.

In this contribution, we provide evidence that retinal indeed influences the protein-lipid interaction. Incorporation of modified retinals led to changes in the protein-lipid interactions, which had long-range character. It is also demonstrated that the retinal influences the extent of the long-range interactions. The effects of retinal on the lipid phase were dependent on the retinal structure in close vicinity to the Shiff base and on the compactness of the protein structure.

Materials and methods

The synthesis and spectral characterization of Ant have been described in the literature (Perochon and Tocanne 1991; Perochon et al. 1991, 1992). The synthesis and characterization properties of the halogenated retinal analogues used in this study are also in the literature (Asato et al. 1978; Asato and Liu 1986).

1,2-Didodecanoyl-*sn*-glycero-3-phosphocholine (Lau), 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (Myr), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (Pam), and 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (Ste) were purchased from Sigma. The PM suspension of *Halobacterium halobium* strain S9 and the PM suspension of the mutant D96N (Wacker, Germany) were used.

Apomembrane was prepared by bleaching the PM or the mutant D96N PM in the presence of hydroxylamine as described in the literature (Hiraki et al. 1987). The regeneration of bR with halogenated retinal analogues was performed according to the earlier described method (Tierno et al. 1990), with one modification. Instead of white membranes the apomembranes were used. The reconstitution procedure and the protein denaturation test were essentially similar to that described by Piknova et al. (1993).

Fluorescence excitation and emission spectra were recorded with a LS 50 Perkin Elmer spectrofluorimeter equipped with a home-made thermostated cuvette holder (temperature stabilization, ± 0.2 °C). λ_{exc} was 340 nm for the emission spectra and λ_{em} was 460 nm for the excitation spectra. Absorption spectra were recorded with a Shimadzu 2000 spectrometer.

Data analysis and calculations were performed with the computer program Prism (GraphPad Software).

Results

Control experiments

The study of the phase behavior of protein/lipid recombinants requires a sensitive method. The approach developed by Piknova et al. (1993) appears to be very efficient in the study of subtle effects. This approach uses the fluorescence and phase properties of the Ant probe to detect gel/liquid phase transitions.

Fluorescence properties of the Ant probe

In order to test the usefulness of Ant as a suitable probe, we tested the influence of bR analogues on Ant fluorescence spectra. Figure 1 presents the fluorescence excitation and emission spectra of Ant in 14-F-bR/Lau recombinants at temperatures of 10 (a), 20 (b), 30 (c), and 40 °C (d). The spectra match the excitation and emission spectra of Ant in bR/Lau vesicles and they are very similar to the spectra of Ant in Lau vesicles without bR (compare fig. 2B, C in Piknowa et al. 1993). These results demonstrate that the presence of the 14-F-bR analogue did not alter the fluorescence properties of Ant. Similar measurements were performed for all samples used. For the sake of clarity, Fig. 1 (dashed lines) presents only one set of curves for Ant in 12-F-bR/Lau vesicles at 10 (a) and 30 °C (c). The excitation spectra were similar to those observed for Ant in 14-F-bR/vesicles. Hence, one can assume that all bR analogues used do not influence Ant fluorescence properties. Both the excitation and emission spectra of Ant can be used for diagnosis of bR/lipid properties. However, the excitation spectra have an advantage over the emission spectra. The temperature does not diminish the structure of the excitation spectra and the potential energy transfer between Ant and bR does not change the mutual relationships between the actual structures (data not shown). Hence, the data can be analyzed with meaningful precision.

Phase properties of the Ant probe

The lipid phase behavior was monitored as a change in the ratio of the Ant excitation spectra measured at two wavelengths as a function of temperature (Piknova et al. 1993). However, instead of registering the changes in the ratio of the Ant excitation spectra at 415 nm and 400 nm (I_{415}/I_{400}), we registered the changes in I_{415}/I_{395}.

Figure 2 (filled circle lines) shows the curves representing the phase transition of lipids in vesicles without any protein. Precise temperature changes (every 1 °C for all samples) and very reproducible results allowed us to

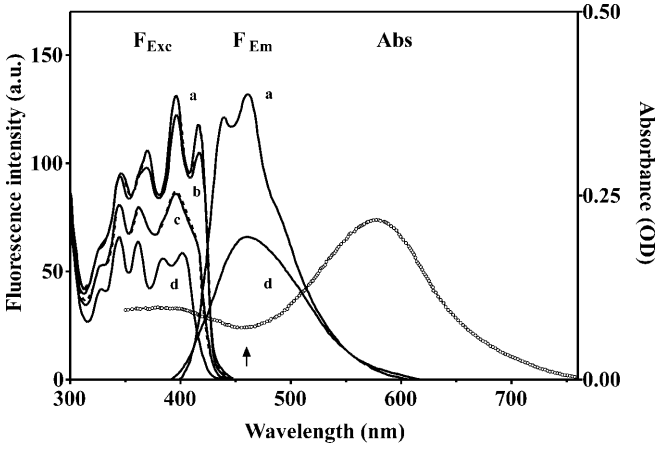

Fig. 1 Fluorescence excitation (F_{Exc}) and emission (F_{Em}) spectra of Ant in lipid vesicles; 0.005 mol/mol Ant (with respect to lipids) in 14-F-bR/Lau 1:80 (mol/mol) recombinants, at temperatures of 10 (*a*), 20 (*b*), 30 (*c*), and 40 °C (*d*). *Dashed lines* represent excitation spectra of Ant vesicles containing 12-F-bR. $\lambda_{exc} = 340$ nm for the emission spectra, $\lambda_{em} = 460$ nm for the excitation spectra. An *arrow* indicates the wavelength for the excitation spectra. The *open circle line* in the Abs region represents the absorption spectrum of 14-F-bR in Lau vesicles. The absorption spectrum refers to the right axes

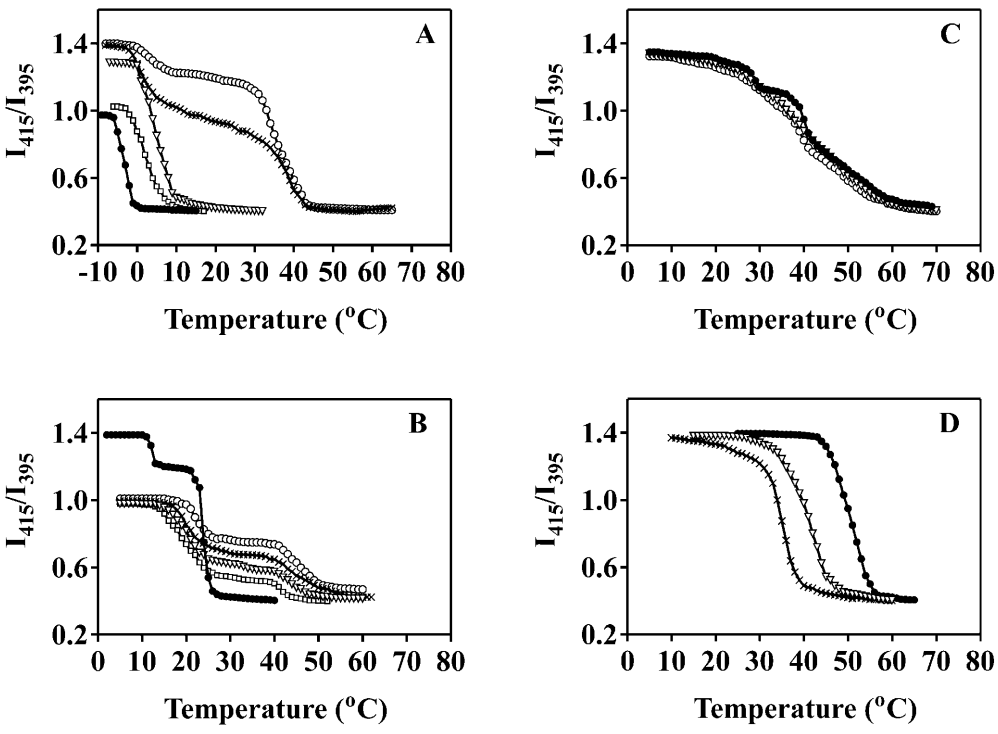

Fig. 2A–D Influence of temperature on the phase state of 12-F-bR/phosphatidylcholine recombinants with different molar ratios. **A** bR/Lau, **B** bR/Myr, **C** bR/Pam, and **D** bR/Ste. bR/phosphatidylcholine molar ratios: (●) 0, (❑) 0.002, (∇) 0.005, (×) 0.01, (○) 0.02

discriminate the characteristic temperatures of the phase transition. The temperature of the gel/liquid phase transition for the pure lipids was about −4 °C for Lau (Fig. 2A), about 12 °C and 23 °C for Myr (Fig. 2B), about 28 °C and 41 °C for Pam (Fig. 2C), and about 50 °C for Ste (Fig. 2D). These values agree well with data previously reported (Lewis et al. 1987).

Influence of 12-(10-,8-)F-bR on the phase transition of lipids

For each of the four lipid species, recombinants with different bR analogues and with different bR/lipid ratios were prepared. The excitation spectra of Ant were recorded at different temperatures. The shape of the curves representing the phase transitions and the values of the transition temperature were very reproducible. Hence, for each lipid species, only three independent registrations were performed.

Changes in the I_{415}/I_{395} ratio with temperature for selected bR/lipid molar ratios are shown in Fig. 2A–D. Although the experiments were performed for vesicles containing 12-(10-,8-)F-bR, the data obtained only with 12-F-bR are presented as representative for experiments with 10- and 8-F samples.

The characteristic changes in the phase curves might be summarized as follows. Addition of bR analogues to Lau resulted in a progressive upward shift in the phase transition temperature of the lipid (Fig. 2A). From biphasic behavior at low bR/Lau ratios, the phase transition became triphasic for a higher bR content. The temperatures of the phase transition were very similar for these samples. For example, for a 0.02 bR/Lau molar ratio the transitions were around 4 °C and 36 °C for 12-F-bR/Lau (Fig. 2A). The respective temperatures were 3 °C and 36 °C for 10-F-bR/Lau and 2 °C and 37 °C for 8-F-bR/Lau (data not shown). Also, for all these samples the ratio I_{415}/I_{395} progressively increased with increasing bR concentration.

The phase transitions of 12-F-bR/Myr are presented in Fig. 2B. The phase transitions were triphasic for all bR/lipid ratios used. An increase of bR/Lau molar ratio from 0.002 to 0.02 induced the two transition temperatures to shift from around 20 to 22 °C and from around 41 to around 45 °C for 12-F-bR/Lau. The respective temperature shifts were 20 to 23 °C and 40 to 44 °C for 10-F-bR/Lau, and 21 to 24 °C and 40 to 44 °C for 8-F-bR/Lau (data not shown).

Addition of bR analogues to Pam did not alter significantly the phase curves of the lipids (Fig. 2C). In all cases, the addition of proteins to the lipids resulted in a slight broadening of the phase transition, which remained centered around the temperature of the pretransition (27–31 °C) and around the temperature of main transition (40–41 °C).

A different effect is induced by bR analogues when incorporated into Ste vesicles. The addition of the protein to the lipids decreased the temperature of the phase transition: the higher the protein concentration, the larger downshift of the biphasic phase transition. Figure 2D presents examples of phase curves for 12-F-bR/Ste vesicles. The downshift is from 50 °C for pure lipids to 40 °C and 34 °C for 0.005 and 0.01 molar ratios of bR/Ste, respectively. Similar effects were observed for all other bR analogues, with temperatures of the phase

transition around those for 12-F-bR/Ste (data not shown).

For all presented data the shapes of the phase curves, the values of the phase transition temperature, and the effects of variable protein concentration for 12-(10-, 8-)bR/lipids used were very similar to results obtained for bR/lipids and agree with results for native bR published by others (Dencher et al. 1983; Lewis and Engelman 1983a; Piknova et al. 1993). Hence, the results confirm the suggestion that bR has a strong influence on the phase transition temperature of the various lipid species in which it is reconstituted, depending on their chain length. These results also provide evidence that the chemical modification of retinal (substitution of the hydrogen atom at the 12-, 10-, or 8-positions with fluorine) did not introduce any specific changes in the protein that might influence the phase transition of the lipids in a manner different from bR effects.

Influence of 14-F-bR and 14-F-D96N on the phase transition of lipids

Experiments similar to those described in the previous section were performed for 14-F-bR/lipid and 14-F-D96N/lipid vesicles. For each of the four lipid species, recombinants with different bR/lipid ratios were prepared and for each of these recombinants the fluorescence excitation spectra of Ant were recorded at different temperatures.

Figure 3 presents typical results of phase curves for 14-F-bR/lipid (solid lines) and for 14-F-D96N/lipid (dashed lines). Generally, both bR analogues had a very strong influence on the lipid phase transition. However, the analogue regenerated with mutated bR (D96N) displayed slightly stronger effects.

Addition of bR and mutated bR, regenerated with 14-F-retinal, to Lau up-shifted the phase transition temperature (Fig. 3A). For example, for a bR/lipid ratio of 0.02, the temperature shift was about 5 °C for 14-F-bR/Lau and about 9 °C for 14-F-D96N/Lau as compared with results for 12-F-bR/Lau (Fig. 2A, and Fig. 3A). The ratios I_{415}/I_{395} progressively increased with increasing bR concentration. However, this increase was faster (especially in the case of 14-F-D96N/Lau) than that for native bR. This suggests that incorporation of 14-F-retinal either into bR opsin or mutated bR opsin led to changes in the protein structure and properties that induced a more efficient increase in the molecular packing of the lipids in the gel phase.

Addition of 14-F-bRs to Myr induced even more pronounced differences in the lipid phase transition (Fig. 3B). The temperatures of the phase transition were again up-shifted with the increase in protein concentration: the pretransition from 21 °C to 24 °C and the main transition from 48 °C to 55 °C. However, the most pronounced changes occured in the temperature region below 20 °C, the gel phase. For 12-(10-, 8-)F-bR and bR, smaller I_{415}/I_{395} ratios were measured in the presence than in the absence of bR. In the case of bR, this was interpreted as bR inducing some loosening of the lipid molecular packing (Piknova et al. 1993). Surprisingly, 14-F-bR and 14-F-D96N did not induce such lipid behavior. The phase curves for higher bR concentrations reached the region defining the gel state faster than the curve for pure Myr, suggesting an

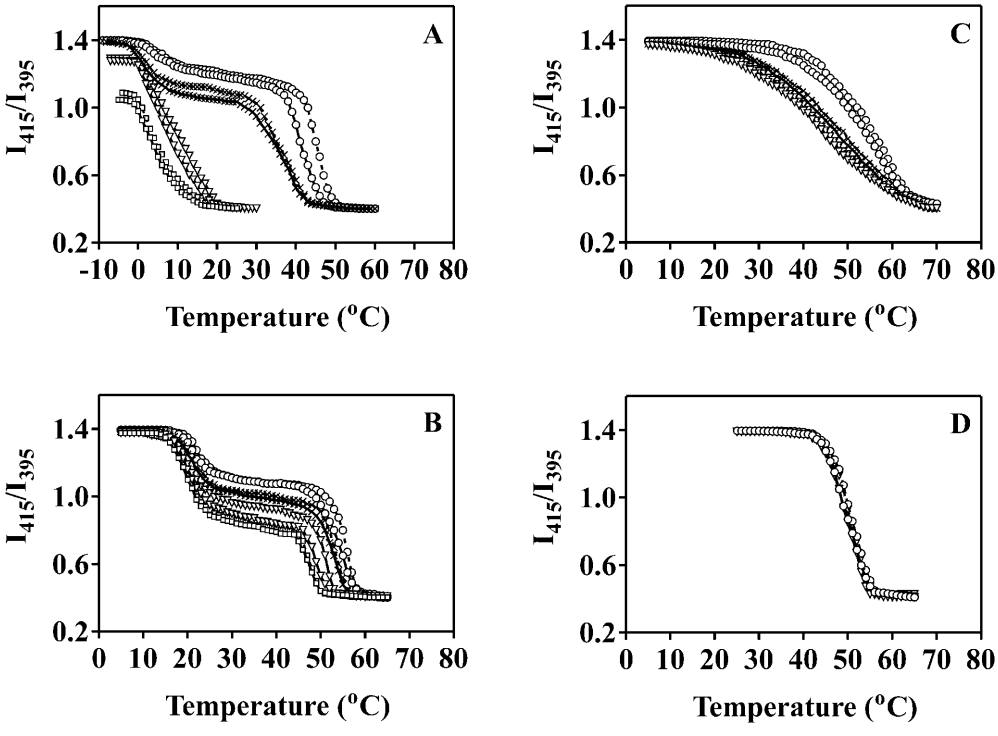

Fig. 3A–D Influence of temperature on the phase state of bR/phosphatidylcholine recombinants with different molar ratios. **A** bR/Lau, **B** bR/Myr, **C** bR/Pam, and **D** bR/Ste. *Solid lines* 14-F-bR, *dashed line* 14-F-D96N. bR/phosphatidylcholine molar ratios: (❑) 0.002, (∇) 0.005, (×) 0.01, (❍) 0.02

increase in the molecular packing of the lipids in the gel phase.

The effects of 14-F-bRs on the Pam phase transition (Fig. 3C) were also different from those observed for previous samples. 12-(10-,8-)F-bR and bR at different concentrations practically had no effect on the Pam phase transition (Fig. 2C). Addition of higher concentrations of 14-F-bRs to Pam resulted in a progressive upward shift in the phase transition temperature of the lipid but keeping the biphasic character of the curves (Fig. 3C). The shift of the temperature was from 42 °C (for a lower concentration of 14-F-bR) to 55 °C (for a higher concentration of 14-F-D96N). These results also may be interpreted as an increase in the molecular packing of the lipids in the gel phase.

A biphasic phase transition was observed in 14-F-bR/Ste or 14-F-D96N/Ste mixtures, with the main phase transitions around 50 °C (Fig. 3D). An increase of the 14-F-bRs concentration did not alter the phase curves, indicating that the protein had no effect on the phase transition. This is contrary to effects caused by 12-(10-, 8-)F-bR and bR. In those cases, the addition of the protein to the lipid decreased the phase transition temperature (Fig. 2D).

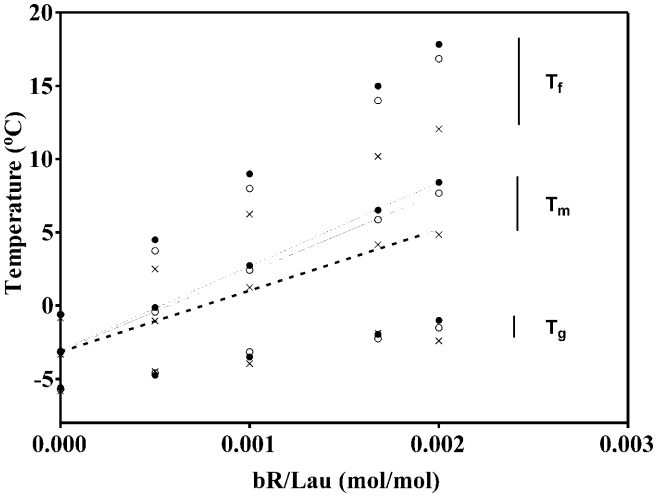

Fig. 4 Phase diagram for bR/Lau. *Dashed line* represents results for 12-F-bR/Lau; *open symbols and solid line* and *full symbols and solid line* correspond to 14-F-bR/Lau and 14-F-D96N/Lau, respectively. T_g and T_f temperatures corresponding respectively to the beginning and the end of the gel/liquid phase transition. $T_m = 0.5(T_g + T_f)$ represents the mean transition temperatures. The T_m lines show the best fitting of Eq. (1) to the experimental data ($\xi = 1.1$ nm for 12-F-bR/Lau, $\xi = 2.0$ nm for 14-F-bR/Lau, and $\xi = 2.2$ nm for 14-F-D96)

Phase diagrams of 12(14)-F-bR/Lau or 14-F-D96N/Lau recombinants

The approach with Ant as a fluorescent probe for the lipid phase transition allowed us to determine both T_g and T_f, the temperatures corresponding to the beginning and the ending of the gel/liquid phase transition, respectively. An increase of protein/lipid ratio up to 0.002 (mol/mol) linearly increased the temperatures (Fig. 4). However, the slope of the linear increase was dependent on the protein used: the smallest for 12-F-bR/Lau (crosses and dashed line), medium for 14-F-bR/Lau (open circles and solid line), and the highest for 14-F-D96N/Lau (filled circles and solid line). The values of $T_m = 0.5(T_g + T_f)$ are also indicated. The shift in the transition temperature will be applied to estimate ξ, the coherence length of the lipid membrane.

Discussion

Since our first experiments with halogenated bR (Tierno et al. 1990) there has been a growing amount of information indicating that these bR analogues might be very useful for investigating the correlation between bR structure and its function via specific substrate-protein interactions (Taiji et al. 1991; Bryl et al. 1994; Gat et al. 1997). However, these results demonstrated rather intrinsic properties of bR. The preliminary results of our further experiments with fluorinated bR suggested that 14-F-bRs might induce long-range effects on the PM (Bryl and Yoshihara 1997). The explanation that the protein has a strong influence on the lipid phase is now quite obvious. However, we used bR analogues in which retinal was modified. Hence, the substitution of the chromophore resulted in observed changes. The question was raised whether retinal can induce changes in the protein-lipid interaction and how to correlate the properties of retinal with the whole protein influence on the lipid phase. In parallel, doubts appeared that the retinal influence might be too small to be detected.

The incorporation of retinal analogues, with changed shape or altered electronic properties, into the binding site of bR has been used to strengthen or to diminish the influence of the protein surroundings. Therefore the demonstration with fluorinated retinal that the chromophore can induce a correct folding of the polypeptide chain of bR (Kollbach et al. 1998) suggested to us that it may be a useful approach in clarification of whether and how retinal can influence the neighboring (and/or bulk) environment.

We have tested different techniques like fluorescence depolarization and differential scanning calorimetry. However, the approach developed by Piknova et al. (1993), which uses the fluorescence and phase properties of the Ant probe, proved to be a very convenient method for studying the protein effect on the lipid phase transition. Also the sensitivity and reproducibility of this method predestine it to study subtle effects like the influence of protein structure on membrane lipids and particularly on long-range protein/lipid interactions.

The results indicate that 12-(10-,8-)bR had a strong influence on the phase transition temperature of the various phosphatidylcholine species in which it was reconstituted, depending on the chain lengths (Figs. 2 and 4). A rigidifying effect was observed on short and me-

dium chain length Lau and Myr molecules (Fig. 2A, B). The phase properties of Pam were not significantly altered (Fig. 2C). In contrast, a fluidifying effect was observed on long-chain Ste molecules (Fig. 2D). Since these results are very similar to those observed for bR/lipids, one can conclude that hydrogen substitution with fluorine atom at the 12-, 10-, or 8-position did not alter the protein structure to such an extent as to influence the phase properties of the lipids.

Surprising effects were observed when retinal in bR or mutant bR was substituted with 14-F-retinal. The distinct changes were related to the stronger up-shift of the phase transition temperature with an increase of bR concentration in the lipid vesicles and to a different correlation between bR-induced phase behavior and the length of the lipid chains (Figs. 3 and 4).

The results suggested a rigidifying effect of 14-F-bRs on short (Lau) and two medium (Myr and Pam) chain length molecules. The phase properties of long-chain Ste molecules were not practically altered. Moreover, no fluidifying effect was observed on any phosphatidylcholine species used.

The influence of bR can be qualitatively accounted for by plotting the differences ΔT between the phase transition temperatures T_m measured in the presence and absence of protein versus the mean hydrophobic thickness (d_m) of the unperturbed bilayer (Riegler and Mohwald 1986; Peschke et al. 1987; Piknova et al. 1993). Figure 5 presents results for 12-F-bR (crosses), 14-F-bR (open circles), and 14-F-D96N (filled circles). We assumed a value of 3.0 nm for the hydrophobic length (d_p) of the protein (Lewis and Engelman 1983a). According to Lewis and Engelman (1983b) and Sperotto and Mouritsen (1988), we used the following values for the hydrophobic thickness of the lipid bilayer in the fluid phase (d_f) and in the gel phase (d_g): 1.95 nm and 2.93 nm for Lau, 2.3 nm and 3.45 nm for Myr, 2.6 nm and 3.9 nm for Pam, and 2.95 nm and 4.43 nm for Ste. The mean hydrophobic thickness was calculated according to $d_m = 0.5(d_f + d_g)$.

Microscopic models suggest that hydrophobic thickness requirements are of prime importance for the protein lateral distribution (Sperotto and Mouritsen 1993). In the case of bR, the energetic content due to the hydrophobic thickness adjustment between the protein and the surrounding lipid bilayer should be lowest in the case of Myr/Pam: the hydrophobic thickness of 3.0 nm for bR is intermediate between the hydrophobic thickness of Myr and Pam (Lewis and Engelman 1983b). It is clearly seen from Fig. 5 that 12-F-bR/Pam represents a similar case in which there is reasonably good matching between the hydrophobic thickness of the protein and Pam and therefore in which the perturbation induced by the protein is minimal. Hence, keeping in mind the assumption about hydrophobic thickness requirements and the hydrophobic thickness of bR ($d_p \approx 3.0$ nm) and Pam ($d_m \approx 3.2$ nm), one can assume that the hydrophobic thickness of 12-F-bR should be between 3.0 and 3.2 nm. This estimation is also confirmed by phase transition

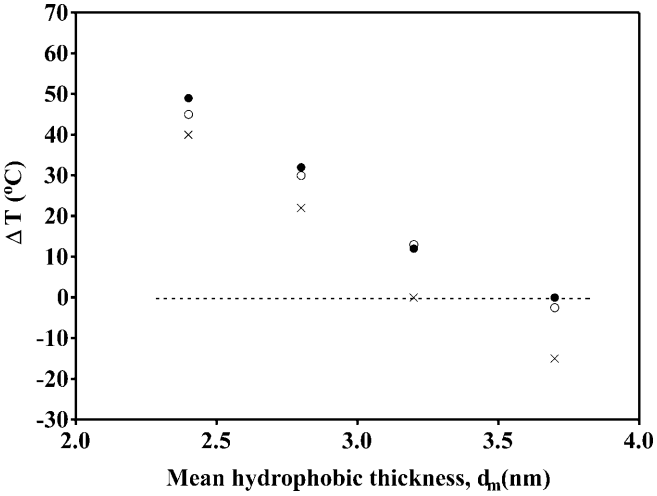

Fig. 5 Plot of ΔT versus the mean hydrophobic thickness of lipid bilayer (d_m) for bR (×), 14-F-bR (○), and 14-F-D96N (●). ΔT is the difference between the mean temperature of the phase transition measured in the presence and absence of proteins for Lau, Myr, Pam and Ste. $d_m = 0.5(d_f + d_g)$, where d_f and d_g are hydrophobic thickness of the lipid bilayer in the fluid phase and in the gel phase, respectively

curves presented in Fig. 2C which are very similar to those for native bR [compare also Piknova et al. (1993), Fig. 3C].

The presence of 14-F-retinal in the protein structure induces a different relationship between ΔT and d_m. An up-shift in the curves is observed for both 14-F-bR and 14-F-D96N. This demonstrates that there should not be a significant mismatch between the hydrophobic thickness of the proteins and the hydrophobic thickness of Ste. Indeed, almost no perturbation induced by 14-F-bRs on Ste was observed (Fig. 3D). Therefore, the shift in the curve (Fig. 5) may suggest a slightly higher hydrophobic thickness for 14-F-bRs, with their values between the hydrophobic thickness of Pam and Ste (3.2–3.7 nm). The hydrophobic thickness of 14-F-D96 seems to be closer to the hydrophobic thickness of Ste.

It is worth noting that, irrespective of imprecise hydrophobic thickness estimations, one fact should be emphasized. The modification of retinal caused the change in the matching of the protein and the lipids. It enlarged the mismatch with short chain length molecules and enlarged the matching with long-chain molecules. These qualitative results may indicate that the active site (like retinal in bR) influences the interaction of the whole protein with the lipids. From other results it is known that proteins may induce long-range lipid-protein interactions (Rehorek et al. 1985). Hence, the question arises as to whether retinal influences only local interactions or may have a contribution in long-range interactions.

Coherence length ξ and bilayer hydrophobic thickness

Sperotto and Mouritsen (1991) have developed a statistical mechanical lattice model which, in the case of

mismatch between the hydrophobic thickness of lipids and proteins in a membrane, enables the hydrophobic lengths of the various lipid layers surrounding the protein and their decay with respect to the distance from the protein to be calculated. The lipid perturbation is expressed by ξ, the coherence length of the lipid membrane.

The coherence length ξ can be estimated from the phase transition temperature shift induced by the increase of protein concentration in the lipid vesicles. The difference between the mean transition temperature $T_m(X_P)$ of the lipid perturbed by the protein and the transition temperature $T_{m,L}$ of the pure lipid can be expressed as follows (Riegler and Mohwald 1986; Piknova et al. 1993):

$$T_m(X_P) - T_{m,L} = 16[\xi\Phi_P/\pi + \xi^2][(d_m - d_p)/(d_{L,f} - d_{L,g})]X_P \quad (1)$$

where d_m, d_f, and d_g are already defined above, X_P is the protein mole fraction in the system, Φ_P is the perimeter of the protein, and d_p is the hydrophobic length of the protein.

The T_m lines in Fig. 4 show the best fitting of Eq. (1) to the experimental data: $\xi = 1.1$ nm for 12-F-bR/Lau, $\xi = 2.0$ nm for 14-F-bR/Lau, and $\xi = 2.2$ nm for 14-F-D96. The value of $\xi = 1.1$ nm for 12-F-bR/Lau corresponds to the results for bR/Lau: $\xi = 1.1$ nm (obtained by us) and $\xi = 1.2$ nm (obtained by Piknova et al. 1993).

The mean lipid hydrophobic thickness, $d_L(I)$, of the nearest layers around one isolated protein are related to the coherence length as (Sperotto and Mouritsen 1991):

$$d_L(I) = d_{L,0} + (d_P - d_{L,0}) \exp(-D(I)/\xi) \quad (2)$$

where $d_{L,0}$ is the mean lipid-chain length of the unperturbed lipid bilayer and $D(I)$ is the distance of the lipid from the protein.

Figure 6 presents the results of the application of Eq. (2) to $\xi = 1.1$ nm for 12-F-bR/Lau, $\xi = 2.0$ nm for 14-F-bR/Lau, and $\xi = 2.2$ nm for 14-F-D96. For the 12-F-bR/Lau system, the 7th to 8th layer from the protein surface corresponds to the bilayer unperturbed hydrophobic thickness. This agrees well with the distance for long-range native bR/lipid interactions determined by Piknova et al. (1993) and Rehorek et al. (1985).

For 14-F-bR(D96N)/Lau, 8 layers from the protein surface are not enough for the bilayer to recover its unperturbed hydrophobic thickness. The influence of 14-F-bRs on the order of the lipid phase extends beyond 10–11 lipid layers.

The proximal end of the chromophore affects protein-lipid interactions

The fluorine atom is highly electronegative. Hence, its substitution for a hydrogen atom in the retinylidene chromophore can affect not only the electronic property of the chromophore but also the interaction of the chromophore with opsin, especially the polarized or charged amino acids residue(s) near the chromophore. Therefore, one can expect that bR analogues with a fluorinated chromophore may provide useful information concerning the role of the chromophore in the structure/function of native bR.

The specific interaction between the fluorine atom and opsin can be discussed in two categories: specific steric interaction and electrostatic interaction. The specific steric interaction may be related to the increase in size caused by replacing a hydrogen atom with a fluorine

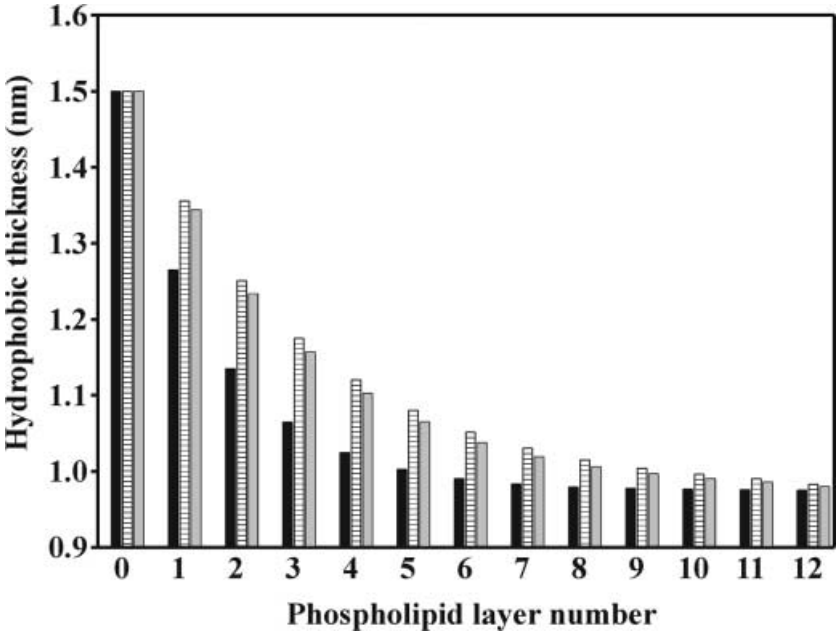

Fig. 6 The decay of the hydrophobic thickness of the Lau bilayer perturbed by 12-F-bR (*solid bars*), 14-F-bR (*dashed bars*), and 14-F-D96N (*bars filled with lines*). The calculation was performed using Eq. (2). The distance is expressed as the number of phospholipid layers from the surface of the protein. The calculations were performed with a mean molecular area of 0.5 nm^2 for the lipid, a protein hydrophobic thickness of $d_p = 3.0$ nm, a mean lipid-chain length of unperturbed lipid bilayer $d_{L,0} = 1.95$ nm, and $\xi = 1.1$ nm for 12-F-bR/Lau, $\xi = 2.0$ nm for 14-F-bR/Lau, and $\xi = 2.2$ nm for 14-F-D96

atom. Both the van der Waals radius of a fluorine atom and the bond distance between the fluorine and carbon atoms are larger than those in the case of a hydrogen atom. The van der Waals radius is 1.35 Å and 1.19 Å for fluorine and hydrogen atoms, respectively. The bond distances are 1.41 Å (F-C) and 1.09 Å (H-C). The increase in van der Waals radius and the bond distance may also strengthen any electrostatic interaction. A fluorine atom is highly electronegative, so the C-F bond may be polarized by inducing a high electron density around the fluorine atom. This high electronegativity may act in two possible ways: the first is an attractive interaction through a hydrogen bond with a neighboring acidic hydrogen such as carboxyl, hydroxyl, thiol, or protonated amino group. The second is a repulsive interaction with a neighboring group bearing excessive electron density, e.g. negatively charged parts of amino groups.

The small increase in size by replacing a hydrogen atom with a fluorine atom in the chromophore seems to introduce no substantial steric interaction to the phenomena observed for unphotolyzed bR. Hence, the electrostatic interactions with groups in the vicinity of the protonated Schiff base should be responsible for the observed effects. Of the four retinal analogues studied, only the replacement of 14-H by a fluorine atom resulted in alterations of bR properties. Therefore, the candidate for the interaction should be positioned within "electrostatic distance" from the 14-F atom and should play an important role in stabilizing the bR, since this local action is sensed relatively "far" from the center of bR, in the lipids surrounding the protein.

According to current models of bR based on crystallographic studies (Henderson et al. 1990; Grigorieff et al. 1996; Pebay-Peyroula et al. 1997; Essen et al. 1998; Luecke et al. 1998, 1999), several residues can fulfill the above-mentioned criteria.

Different biophysical studies have established that in the light-adapted state of bR the protonated Schiff base is stabilized by a diffuse counterion, presumably involving a H-bonded complex of water with the positively charged Arg82 side chain and the deprotonated side chains of Asp212 and Asp85. Following data obtained by Griffiths et al. (2000), Grigorieff et al. (1996), and Luecke et al. (1998) the distances (in Å) of Asp212 to the 14-C-retinal are 4.4, 4.29, and 4.4, respectively. The distances of Asp85 to 14-C-retinal are the following: >6.0, 5.13, and <6.0. In all cases, the side chain of Asp212 is held in position by interactions with the side chains of Tyr57 and Tyr185, with a distance to the 14-C-retinal of <4.8 Å and <4.3 Å, respectively. These data suggest that Asp212 and/or Tyr185 can be regarded as potential candidates for a strong interaction with the 14-F atom.

However, other groups may interact directly or indirectly with the 14-F atom. Thr89 is located one turn above the Asp85 counterion on the C-helix. In a model of bR based on electron diffraction (Grigorieff et al. 1996) the hydroxyl oxygen of Thr89 was found to be located about 3 Å from Asp85 and 3.8 Å from the nitrogen of the Shiff base. Thus, Thr89 is in a good position to participate directly in the active site of bR and to interact with other (via a hydrogen bond with Asp85, for example) residues in the retinal binding pocket (Rothschild et al. 1992; Russel et al. 1997). The indole N-H of Trp86 is near the carboxylate of Asp85 (Hatanaka et al. 1997). Thus, Trp86 may be involved in binding the water molecules, forming a network of hydrogen bonds and participating in the interaction with 14-F-retinal. Arg82 points toward the extracellular side and the distance between the guanidinium group of Arg82 and the Schiff base nitrogen is 11.5 Å. However, a molecular dynamics simulation on bR demonstrated that Arg82 might be located at a distance of 4.5 Å from the protonated Schiff base (PSB) (Nagel et al. 1997) forming the complex counterion of the PSB (Petkova et al. 1999). Trp86 is hydrogen bonded to Asp85 and participates in the complex counterion of the Schiff base with Asp85, Arg82, and Asp212 (Oesterhelt 1998).

All of mentioned amino acids fulfill conditions to be good partners for interaction with the 14-F atom. However, if we accept that Asp212 is located about 4.4 Å from C-14-retinal, that 1.35 Å is the van der Waals radius for fluorine, and that the bond distance F-C is 1.41 Å, these values give 2.99 Å as the distance between Asp212 and the fluorine atom, a good distance for a strong electrostatic interaction. Hence, Asp212 (together with Tyr185) seems to be a very probable candidate for strong interaction with the 14-F atom.

The plausible mechanism of changes in bR structure induced by 14-F might be as follows. It was demonstrated (Luecke et al. 1998, 1999) that Tyr185 and Asp212 form a polarizable hydrogen bond. This H-bond might act to stabilize the bR binding pocket and especially the orientations of the F and G helices. The incorporation of the F atom at the 14-position of retinal may cause disruption (or distortion) of this bond via a small downward shift of helix G (which carries Asp212) or a shift of helix F (which carries Tyr185), or via a change in the angle that the OH bond makes with the COO^- group (Rothschild et al. 1990). The disruption of the H-bond via a small (about 1.5 Å) downward shift of helix G was suggested in order to explain altered stabilities of the conformations in Asp212 mutants (Rothschild et al. 1990). The shift of one helix (G or F) may induce a shift of another one owing to strong cooperation between them (Oesterhelt et al. 2000).

If the assumption about the slight shift of the G helix (and/or F helix) is correct, it will be tempting to compare this action with the side view of the bR and ordered lipids (Luecke et al. 1999). The extracellular portion of the protein is more completely embedded in the hydrophobic bilayer than in the cytoplasmic region. Hence, even a small shift of the helix towards the extracellular side should have stronger consequences on the hydrophobic mismatch.

The interaction of 14-F with opsin was strengthen by substitution of aspartic acid 96 with asparagines. This

puzzling result suggests an involvement of Asp96 in the changes in the opsin region close to the proximal end of the chromophore. However, Asp96 is far (more than 11 Å) from the place of investigation (Grigorieff et al. 1996). Therefore, it might be difficult to explain the direct interaction between 14-F and Asp96. Surprisingly, this group is functionally linked with groups in close vicinity of the Schiff base and influences their properties (Heberle et al. 1993; Rothschild et al. 1993; Riesle et al. 1996; Kandori et al. 2000). Particularly, the mutation of D96 introduces a local electrostatic change which is the cause of the large-scale protein conformational shift in bR (Brown et al. 1997) and may disturb the tight packing of bR (Kikukawa et al. 1995, 1997). It seems very probable that these structural changes may facilitate the electrostatic interaction between the 14-F atom and certain groups like Asp212 (and/or Tyr185), leading to slightly bigger shifts of helix G (and/or helix F) than that in bR.

The hypothetical shift of the helices may be interpreted as a slight change of the hydrophobic thickness of the opsin part. bR, via a hydrophobic matching principle related to the difference in lipid bilayer hydrophobic thickness and protein hydrophobic length, can perform the ordering of the lipids (Sperotto and Mouritsen 1993; Dumas et al. 1997), as was observed in our experiments.

The nature of the study required it to take place in a model system in which bRs were reconstituted with four types of lipids differing in hydrophobic lengths. Each experiment was performed with one type of lipid. However, it must be considered that in real biological membranes there is a large distribution of lipids varying in acyl chain lengths and that, for a given transmembrane protein, the various constitutive α-helices can vary in length and tilting angle with respect to the bilayer normal. Therefore, the possibility that bR, via a hydrophobic matching principle related to the difference in lipid bilayer hydrophobic thickness and protein hydrophobic length, can perform molecular sorting of the lipids at the lipid-protein interface (Dumas et al. 1997) may be generalized by postulating that the protein is surrounded by more than one lipid species because each of its α-helices may select the type of lipid molecule whose acyl chains best match the actual helix length and tilting angle. The results presented indicate that this matching can be extended over the closest to the protein lipid layer and the chromophoric reactive center may influence this process.

An additional aspect of the native PM should be emphasized. In the native PM, bR is organized in a two-dimensional hexagonal lattice of protein trimers (Henderson and Unwin 1975; Hendeson et al. 1986). The essential role of specific PM lipids in 2D-array formation has been emphasized (Watts 1995; Watts et al. 1995). Moreover, the importance of PM lipids in bR photocycle behavior has been suggested (Dracheva et al. 1996; Joshi et al. 1998), although it has been proposed that the bR trimer-membrane interaction rather than 2D-array formation is important in controlling the bR photocycle (Mukhopadhyay et al. 1994, 1996). It is generally agreed that circular dichroism (CD) spectra of bR are strongly indicative of its degree of aggregation (Cassim 1992). The CD spectra of reconstituted bRs were similar to the CD spectra of native bR and fluorine bR analogues obtained by Kollbach et al. (1998) and demonstrated reversible changes in the protein aggregation state upon temperature changes (data not shown). It seems that this aspect of bR contact with lipids is important in examination of bR-lipid interactions (Heyn et al. 1981b; Dencher et al. 1983; Dencher 1986).

The results obtained in a model system indicate interesting actions of the chromophore that can exist in the native membrane. The substitution of the hydrogen atom was related to one specific place of the chromophore. This substitution caused local deformation in opsin. However, the observed effects were related to changes in the protein-lipid interactions. Hence, they were induced relatively far from the place of action, far enough to be called "long-range protein-lipid interactions" (Rehorek et al. 1985), and to postulate that retinal may influence these interactions.

The results also suggest a possible hierarchy in the contribution to resultant protein-lipid interactions: a major contribution induced by the protein part of bR and a minor contribution from retinal, the bR reactive center. The ratio between the major and minor contributions might be altered by changing the structure of both retinal (seen in the 14-F analogues) and protein (seen in the bR mutant). It is important to emphasize that the results suggest that not whole retinal and not all protein parts contribute equally to long-range effects. The extent of these contributions was dependent on the retinal structure in the close vicinity to the Shiff base (proximal end of the chromophore) and on the amino acids that influence the structure of the protein part, like compactness of the protein in the D96N mutant (Kikukawa et al. 1995, 1997).

The actual mechanisms by which the protein modifies the lipid domain are not clear. Because of the increase in the surrounding lipid acyl chain order caused by transmembrane proteins (Heyn et al. 1981a, b; Almeida et al.1992; Piknova et al. 1993), the accumulation of bR at the lipid-phase boundary probably stabilizes the interface and should tend to decrease the purple domain size, leading to an increase of local charge density (Schram and Thompson 1997). Retinal, as a central part of bR, stabilizing bR and inducing tight folding of the peptide chain around itself, may indirectly contribute to these phenomena.

The results demonstrate that retinal as a central active site of bR may influence not only local bR structure but long-range bR properties. It may mean that retinal plays a double role. One role is obvious: direct involvement in proton pumping through a membrane-proton transfer reaction across the PM (Heberle 2000). Another role might be related to participation in the function of whole PM segment-proton transfer reaction along the PM (Heberle 2000).

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