Effects of Lipid Environment on the Light-Induced Conformational Changes of Rhodopsin. 2. Roles of Lipid Chain Length, Unsaturation, and Phase State†

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ABSTRACT: When rhodopsin is incorporated into the saturated short-chain phospholipid dimyristoylphosphatidylcholine, photolysis of the protein results in an abnormal sequence of spectral transitions, and the dominant product of metarhodopsin I decay is free retinal plus opsin [Baldwin, P. A., & Hubbell, W. L. (1985) *Biochemistry* (preceding paper in this issue)]. By incorporation of rhodopsin into a series of phosphatidylcholines of defined composition, we have determined the properties of the lipid environment that are responsible for the altered spectral behavior. Metarhodopsin II is not found in appreciable amounts in bilayers containing acyl chains that are too short (14 or fewer carbon atoms in length), in the presence of only *n*-alkyl chains, or below the characteristic phase-transition temperature of recombinant membranes. Double bonds are not required for the formation of the metarhodopsin II intermediate, as it is observed in diphytanoylphosphatidylcholine recombinants.

The metarhodopsin I (meta I)[1] to meta II spectral transition in ROS membranes involves a conformational rearrangement of the rhodopsin molecule. However, when rhodopsin is incorporated into the saturated, short-chain phospholipid di-14:0-PC, the normal conformational change as judged by spectral transitions does not occur, and the dominant product of meta I decay is free retinal plus opsin (Baldwin & Hubbell, 1985). The object of the work presented here is to determine what properties of the lipid environment are involved in producing the altered spectral (and presumably conformational) change of the protein.

Obvious differences between the di-14:0-PC bilayer and the native lipid environment that may be important are the nature of the lipid head group, the molecular heterogeneity of the bilayer, the degree and type of unsaturation of the acyl chains, the length of the acyl chains (bilayer thickness), and the phase state of the membrane. Which of these differences are important can be tested by preparing rhodopsin recombinants from a series of lipids of defined composition and determining the effect of alterations in the lipid environment on the light-induced conformational changes of rhodopsin. The results of such experiments are presented here. As was discussed in the preceding paper (Baldwin & Hubbell, 1985), a normal meta I to meta II transition is not observed in preparations where rhodopsin is highly aggregated ("insoluble" in the two-dimensional bilayer solvent). Thus, before any observed abnormal rhodopsin behavior can be interpreted as resulting directly from an altered bilayer–protein interaction, the distribution of rhodopsin in the recombinant membrane must be established. For this purpose, freeze–fracture electron microscopy results are reported for recombinant membrane systems that have not been previously examined. In addition, it is important to determine the structural integrity of rhodopsin in the recombinant membranes in order to ensure that the observed spectral behavior is not a result of protein denaturation. The criterion used in the present work is the ability of the bleached protein to recombine with exogenously added 11-*cis*-retinal, i.e., the regeneration yield.

The importance of the first two of the above-mentioned differences between di-14:0-PC and the native lipid can be assessed on the basis of a previous study of one recombinant membrane system performed by O'Brien et al. (1977). In their work, rhodopsin incorporated into recombinant membranes with di-(18:1)$^{c\Delta 9}$-PC underwent the transition from meta I to meta II after illumination, although the transition rate was slightly slower than that observed in the ROS disk membranes. This demonstrates that no specific lipid head group other than phosphatidylcholine is necessary for the occurrence of a normal meta I to meta II transition. In addition, although the native lipid is heterogeneous in two ways, i.e., there are many different head groups and also different types of acyl chains attached to a given head group, this heterogeneity is not a necessity since a normal transition occurs in the presence of a single type of head group and acyl chain. That a normal transition does occur in di-(18:1)$^{c\Delta 9}$-PC, which both is longer than di-14:0-PC and contains a cis double bond, suggests that either a minimum acyl chain length or unsaturation (or both) may be required for the occurrence of normal protein behavior. The experiments presented here explore the effect of these two factors on the behavior of rhodopsin. In addition, the role of the phase state of the membrane is examined.

Experimental Procedures

Materials. Diphytanoyl-PC was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Di-(14:1)$^{c\Delta 9}$-PC was purchased from P-L Biochemicals (Milwaukee, WI). Di-

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[1] Abbreviations: meta I, metarhodopsin I; meta II, metarhodopsin II; ROS, rod outer segment; di-14:0-PC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; di-(18:1)$^{c\Delta 9}$-PC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; diphytanoyl-PC, 1,2-diphytanoyl-3-*sn*-phosphatidylcholine; di-(18:1)$^{t\Delta 9}$-PC, 1,2-dielaidoyl-3-*sn*-phosphatidylcholine; di-16:0-PC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DTAB, dodecyltrimethylammonium bromide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; meta III, metarhodopsin III.

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(14:1)$^{t\Delta 9}$-PC (A grade) and di-16:0-PC (A grade) were obtained from Calbiochem-Behring Corp. Frozen bovine retinas, hydroxylapatite, and DTAB were obtained as described previously (Baldwin & Hubbell, 1985).

Preparation of Recombinant Membranes. Rhodopsin was isolated from frozen dark-adapted bovine retinas and purified by chromatography on hydroxylapatite as previously described (Baldwin & Hubbell, 1985). In order to form the recombinant membranes, purified and delipidated rhodopsin in detergent was combined with a solution of the appropriate lipid dissolved in 2–3 mL of 100 mM DTAB, 15 mM sodium phosphate, and 1 mM DTT, pH 6.6, to give a lipid:rhodopsin molar ratio of 100:1. For some of the lipids, it was necessary to add a small amount of solid DTAB to the solution (before combining it with the rhodopsin) in order to dissolve the lipid completely. The recombinant membranes were then formed by dialysis and harvested as described previously (Baldwin & Hubbell, 1985). The phospholipid content for some of the samples was evaluated by phosphate analysis and was found to be consistent with the above ratio.

Absorption Spectroscopy. Recombinant membranes were prepared for spectroscopy and absorption spectra were taken as previously described (Baldwin & Hubbell, 1985). The pH of the sample was sometimes altered after illumination as previously described (Baldwin & Hubbell, 1985).

Regeneration of Rhodopsin from Bleached Recombinant Membranes. Regeneration was performed according to Hong & Hubbell (1973) at room temperature on samples that had been prepared as for absorption spectroscopy except that the annealing and sonication steps were omitted. Some samples were regenerated in the presence of 1 mM DTT and 1 mM EDTA. In one experiment, the di-16:0-PC recombinant was heated to 44 °C, bleached for 10 s with white light, and incubated at 44 °C for 15 min before regeneration, which was performed at room temperature as described above.

Freeze–Fracture Electron Microscopy. Samples for freeze–fracture were dialyzed against 10 mM MOPS at pH 6.6 during the formation of the recombinant membranes. The last change of dialysis buffer was 10 mM MOPS, pH 6.5. The membrane suspension was concentrated by centrifugation. A drop of the concentrated suspension was placed on a 3-mm diameter paper disk at room temperature and then rapidly plunged into Freon 22 cooled by a liquid nitrogen bath. This procedure was carried out either under dim red light or under room lights after illumination of the sample for 1 min with a 200-W projection lamp equipped with a heat filter. Frozen samples were fractured and shadowed in a Balzers apparatus at −111 °C without etching. Replicas were cleaned in commercial bleach and 20% H_2SO_4 and mounted on bare 400-mesh copper grids. Electron micrographs were taken with a JEOL 100-CX.

Results

Regeneration of Rhodopsin in Bleached Recombinant Membranes. The results for the regeneration of photolyzed rhodopsin in the various recombinant membranes at room temperature in the presence of 1 mM DTT and 1 mM EDTA after addition of 11-*cis*-retinal are presented in Table I. The regeneration yields of all the recombinants used for this study are high, and these lipids apparently provide a membrane environment consistent with the stability of opsin at room temperature. Since the study of the behavior of rhodopsin in fluid-phase di-16:0-PC recombinant membrane was carried out at 44 °C, it was important to determine whether denaturation of the protein occurred after photolysis at this elevated temperature. Di-16:0-PC recombinants were heated to 44 °C, bleached, and allowed to thermally decay for 15 min at 44 °C. Subsequent regeneration at room temperature gave a regeneration yield of 50% for rhodopsin in the di-16:0-PC recombinants after this treatment. The omission of DTT and EDTA made little difference to the regeneration yields reported here for the recombinant membranes. Only in the case of the native ROS membranes was a slightly lower regeneration yield obtained when the DTT and EDTA were not included. This is noted because the absorption spectra and freeze–fracture electron micrographs were obtained from samples in the absence of these reagents.

Table I: Properties of Recombinant Membranes

lipid in recombinant	T_m (°C)[a]	T_c (°C)[b]	regeneration yield (%)
di-(14:1)$^{c\Delta 9}$-PC	−50[c]		66
di-14:0-PC	22[d]	26[d]	75[d]
di-16:0-PC	40[d]	40[d]	74
diphytanoyl-PC	−120[e]		85
di-(18:1)$^{c\Delta 9}$-PC	12[f]	10[d]	81[d]
native ROS membranes			97

[a] T_m is the phase-transition temperature of the pure lipid. [b] T_c is the characteristic temperature of the phase transition in the recombinant membrane. [c] Linear extrapolation based on the values of T_m for the phosphatidylcholines di-(18:1)$^{c\Delta 9}$-PC (Barton & Gunstone, 1975), di-(17:1)$^{c\Delta 9}$-PC (Salvati et al., 1979), and di-(16:1)$^{c\Delta 9}$-PC (Van Dijck et al., 1976). [d] Chen & Hubbell, 1973. [e] Lindsey et al., 1979. [f] Wu & McConnell, 1979.

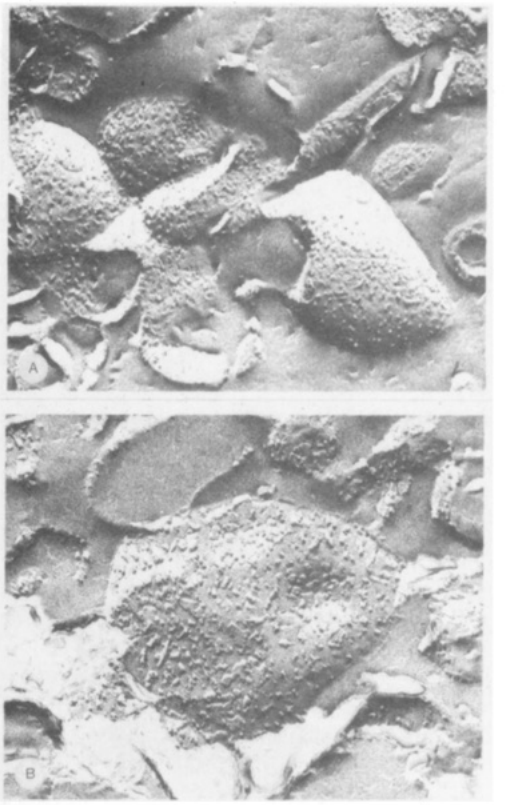

FIGURE 1: Freeze–fracture electron micrographs of recombinant membranes frozen from 20 °C in the dark: (A) rhodopsin–di-(14:1)$^{c\Delta 9}$-PC recombinants (33800×); (B) rhodopsin–diphytanoyl-PC recombinants (42900×).

Two-Dimensional Distribution of Rhodopsin in Recombinant Membranes. Freeze–fracture electron microscopy studies have previously been performed on di-14:0-PC, di-16:0-PC, and di-(18:1)$^{t\Delta 9}$-PC recombinant membranes, and the two-dimensional distribution of rhodopsin in these systems has been determined (Chen & Hubbell, 1973). Recombinants with di-(14:1)$^{c\Delta 9}$-PC and diphytanoyl-PC have not been previously

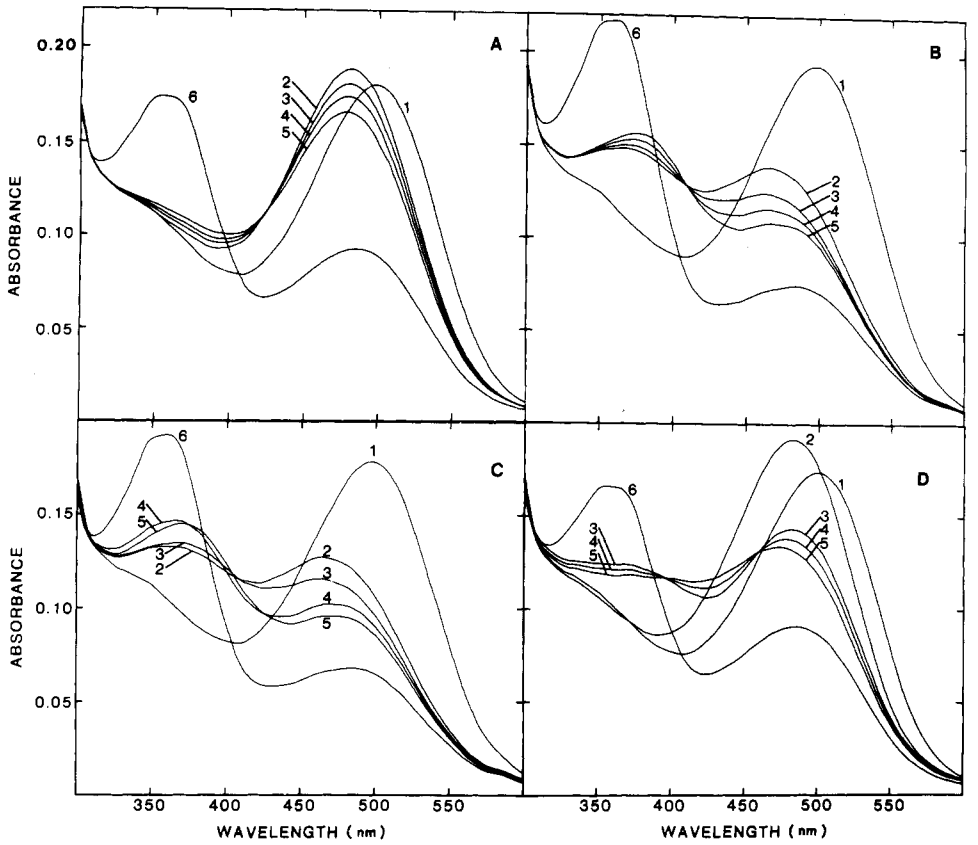

FIGURE 2: Absorption spectra of suspensions of di-(14:1)$^{c\Delta 9}$-PC recombinant membranes in 0.1 M KCl–0.01 M MOPS, pH 6.5. In panels A (15 °C) and B (30 °C), curve 1 was taken before illumination, and curves 2–5 were scanned starting at about 1, 7, 15 and 30 min after illumination, respectively. Curve 6, scanned in the presence of 0.05 M hydroxylamine added after illumination, shows the amount of rhodopsin plus isorhodopsin remaining in the sample. Panel C shows the effect of an increase in pH on the spectral intermediates observed after photolysis at 30 °C. Curve 1 was recorded before illumination at pH 6.5, and curves 2 and 3 were taken starting 2 and 7 min after illumination. After the recording of curve 3, the pH was raised to ≈7.5, and curve 4 was taken 10 min after illumination. Addition of base apparently resulted in the production of *N*-retinylideneopsin 365, which has previously been observed as a product of meta I decay in digitonin-solubilized ROS membranes under basic conditions (Ostroy, 1974). Curve 5 was taken 30 min after illumination and shows a shift of the *N*-retinyldeneopsin 365 peak to longer wavelength, perhaps as a result of further production of free retinal. Curve 6 is in the presence of 0.05 M hydroxylamine added after the preceding spectra were taken. Panel D demonstrates the effect of a decrease in pH on the spectral intermediates observed after photolysis at 2.4 °C. Curve 1 was recorded before illumination at pH 6.5, and curve 2 was scanned starting 1 min after illumination was started. Spectra taken after 4 and 7 min were identical with curve 2. Nine minutes after illumination, the pH was lowered to ≈5.5. Curves 3–5 were recorded 10, 16, and 30 min after illumination, respectively. Curve 6 is in the presence of 0.05 M hydroxylamine added after the preceding spectra were obtained.

studied, and freeze–fracture electron micrographs from these recombinants are shown in Figure 1. Di-(14:1)$^{c\Delta 9}$-PC recombinant membranes that had been rapidly frozen from 20 °C and then fractured are shown in Figure 1A. In these membranes, which were frozen from very far above their characteristic thermal transition temperature, rhodopsin appears to be fairly uniformly distributed, i.e., not aggregated. No major differences were detected between bleached and unbleached samples (data not shown).

Figure 1B is an electron micrograph of diphytanoyl-PC recombinant membranes that were rapidly frozen from 20 °C prior to fracture. Rhodopsin again appears to be reasonably uniformly distributed in this lipid, which has no detectable transition to a solid phase above −120 °C (Lindsey et al., 1979). Once again, no major differences are detected between membranes containing bleached or unbleached rhodopsin (data not shown).

Spectral Transitions Observed after Photolysis of Rhodopsin in Recombinant Membranes. The effect of alterations in the lipid environment on the light-induced spectral changes of rhodopsin was studied for a series of recombinant membranes. In the previous paper, we investigated the characteristic spectral changes of photolyzed rhodopsin in di-14:0-PC bilayers and confirmed earlier work showing that a meta I like species ($\lambda_{max} \approx 475$ nm) was a predominant and stable protein form both above and below the phase transition (O'Brien et al., 1977). In addition, we showed that the decay of this species produced predominantly free retinal ($\lambda_{max} \approx 380$ nm) rather than the expected meta II (λ_{max} also about 380 nm).

In studying the spectral transitions and characteristics of rhodopsin in recombinants formed from a variety of different phospholipids, we find that the general behavior can be simply classified in one of two categories: either similar to the di-14:0-PC recombinants with suppressed meta II formation or similar to the native system with an observable meta II to meta III transition. Thus, in the results presented below, we will compare the spectral transitions and characteristics of the recombinants to those of di-14:0-PC or the native membranes, both discussed in the preceeding paper. These results are presented here in order of increasing acyl chain length of the lipid used for each recombinant.

In its pure state, di-(14:1)$^{c\Delta 9}$-PC has a phase-transition temperature very far below 0 °C (Table I). Thus, near room temperature, the acyl chains of recombinant membranes made with this lipid are in a very fluid state. However, as shown in parts A and B of Figure 2, illumination of di-(14:1)$^{c\Delta 9}$-PC recombinants at 15 and 30 °C produces spectral transitions very similar to those seen for di-14:0-PC recombinants

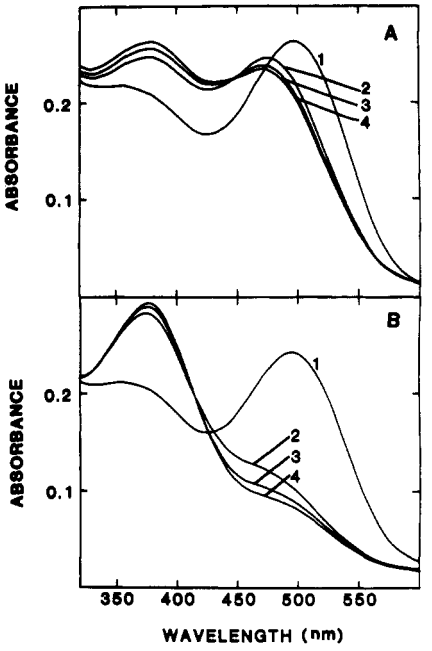

FIGURE 3: Absorption spectra of a suspension of di-16:0-PC recombinant membranes in 0.01 M MOPS, pH 6.8: (A) 24 °C; (B) 44 °C. Curve 1 was taken before illumination. Curves 2– and 4 were scanned starting at 4, 12.5, (8 min for B), and 30 min after illumination, respectively.

(Baldwin & Hubbell, 1985) and quite distinct from the native membrane. As is the case for di-14:0-PC, addition of base to the di-(14:1)$^{c\Delta 9}$-PC recombinants does not cause an increase in the amount of meta I (Figure 2C). Acidification of the di-(14:0)$^{c\Delta 9}$-PC recombinants at 3 °C (Figure 2D) produces a decay pattern very similar to that observed by Ostroy (1974) for digitonin-solubilized ROS membranes under slightly acidic conditions. Ostroy observed that under these conditions, in digitonin, meta II decayed to both *N*-retinylideneopsin 440 and meta III as evidenced by the isosbestic points at 400 and 460 nm and the shift to shorter wavelength of the meta I peak. The occurrence of this same decay pattern in the di-(14:1)$^{c\Delta 9}$ recombinants suggests that, as is the case for the di-14:0-PC recombinants, meta II may make an appearance under acidic conditions above the characteristic thermal transition.

Rhodopsin in di-16:0-PC recombinant membranes also behaves similarly to that in di-14:0-PC recombinants after illumination with respect to spectral transitions. Both below and above the characteristic phase-transition temperature for this system, there is a decrease in absorbance at $\lambda \approx 475$ nm and a concomitant rise at $\lambda \approx 380$ nm (Figure 3). Due to the rather high phase-transition temperature of this lipid (near 40 °C), it is possible that, above the phase transition, the kinetics of meta II appearance and decay are rapid enough that meta II has already decayed before the first postillumination spectrum was taken in Figure 3B. However, observation of the 380-nm absorbance starting 5 s after a 1-s illumination showed only an increase in absorbance at this wavelength and no decrease that could be attributable to the decay of a meta II species.

The changes in absorption spectra observed upon illumination of recombinant membranes made with diphytanoyl-PC (phytanic acid = 3,7,11,15-tetramethylpalmitic acid) are shown in parts A and B of Figure 4 for 15 and 30 °C, respectively. The series of spectral intermediates observed after illumination is strikingly similar to that seen for native ROS membranes [compare with Baldwin & Hubbell (1985), Figure 1A,C]. However, the quantitative details of the transition appear to be altered in this lipid environment; in particular, the ratio of meta I to meta II initially observed is larger, and the meta II decay rate is slower in the recombinant membrane. It is important to note that the 380-nm species observed in diphytanoyl-PC recombinants has the properties of meta II, not free retinal. For example, as is expected for meta II, a change in the pH of the surrounding medium changes its equilibrium with meta I in the diphytanoyl-PC recombinants (Figure 4C,D) as is observed in the native membranes.

The changes in absorption spectra observed upon illumination of di-(18:1)$^{t\Delta 9}$-PC recombinant membranes under several different conditions are shown in Figure 5. At pH 6.5 and 25 °C, which is above the characteristic thermal transition temperature for this system (Table I), the observed spectral intermediates show the same pattern observed in the native system (Figure 5A). At longer times (from 15 to 30 min), meta III decays, and the absorbance at 380 nm increases, probably due to the production of free retinal. As was the case for the diphytanoyl recombinants, the exact details of the transition appear to be altered, and the amount of meta II produced initially is smaller, and the kinetics of its decay are slower than those seen for the native membranes. Below the characteristic temperature, at pH 6.5 and 5.6 °C, a series of spectra similar to those observed for di-14:0-PC recombinants is obtained; e.g., a $\lambda_{max} \approx 480$ nm appears and decays slowly in time with a concomitant rise in absorbance at $\lambda_{max} \approx 380$ nm. Addition of base after photolysis under these conditions does not result in the reappearance of meta I (data not shown). This confirms that the pattern of spectral decay observed below T_c in the di-(18:1)$^{t\Delta 9}$-PC recombinants is similar to that seen for di-14:0-PC (Figures 1B and 2C; Baldwin & Hubbell, 1985) and is not simply a result of a temperature-dependent shift in the meta I/meta II ratio as is observed in the native membranes (Figures 1A and 2A; Baldwin & Hubbell, 1985). Although we have not studied the behavior of this system as a function of temperature through the phase transition, it is clear that the spectral properties are dramatically different above and below the transition temperature. This is the only lipid for which this interesting behavior was observed. Figure 5C shows the results of illumination of di-(18:1)$^{t\Delta 9}$-PC recombinants below their characteristic temperature at pH 5.0. Even though below the characteristic temperature, at this lower pH a more normal spectral decay pattern is observed as meta II decays to meta III followed by the apparent eventual production of *N*-retinylideneopsin 440 (curve 5) as was seen with the di-(14:1)$^{c\Delta 9}$-PC recombinants at acidic pH.

Discussion

Properties of Rhodopsin in Recombinant Membranes. Rhodopsin in native ROS disk membranes exhibits a high regenerability. The regeneration yields for rhodopsin in the recombinant membranes used in this study are also high as shown in Table I. On the basis of this criterion as an assay for the structural integrity of the protein, it appears that all of these lipids provide an environment consistent with the stability of opsin near room temperature, and thus, the observed spectral behavior is not due to massive protein denaturation. A possible exception to this is the di-16:0-PC recombinant above its phase-transition temperature of 40 °C. However, even after bleaching and incubation at 44 °C, 50% of the rhodopsin in this recombinant was still regenerable. Thus, although some of the 380-nm absorbance that appears after photolysis of di-16:0-PC recombinants may be a result of protein denaturation; this is clearly *not* the sole cause.

On the basis of the results of the freeze–fracture electron microscopy presented here and the previous study by Chen &

Hubbell (1973), it is evident that the major determinant of the two-dimensional distribution of rhodopsin in the recombinant membranes is the phase of the phospholipid bilayer. Rhodopsin has now been examined above the characteristic thermal transition temperature in recombinants made from di-10:0-PC, di-12:0-PC, di-(14:1)$^{c\Delta 9}$-PC, di-14:0-PC, di-16:0-PC, diphytanoyl-PC, and di-(18:1)$^{t\Delta 9}$-PC. Dark-adapted rhodopsin appears uniformly dispersed in the bilayer in each of these systems with the exception of di-(18:1)$^{t\Delta 9}$-PC. In this lipid at about 10 °C above T_c, small, but definite, rhodopsin-rich regions are observed (Chen & Hubbell, 1973). When the temperature from which the recombinants are frozen is increased to 37 °C, the particle-rich regions are largely dispersed, but the distribution of rhodopsin does not appear to be completely random (Chen & Hubbell, 1973). It is possible that the small but detectable degree of protein clustering is due to insufficiently fast freezing rates (compared to lipid crystallization rate) in preparing the samples.

The results obtained here and earlier (Chen & Hubbell, 1978) for rhodopsin show the same general trend that was observed in freeze–fracture electron microscopy studies of bacteriorhodopsin in recombinants above their characteristic thermal transitions (Lewis & Engelman, 1983a). Bacteriorhodopsin remains freely dispersed in all the recombinants studied except for those with the longest [di-(24:1)$^{c\Delta 15}$-PC] and shortest (di-10:0-PC) acyl chains. Apparently, for both rhodopsin and bacteriorhodopsin, large differences between the hydrophobic thickness of the protein and the hydrophobic thickness of a particular lipid bilayer can be accommodated without protein aggregation. It seems unlikely that the protein is accommodated in bilayers thinner than natural by exposure of the hydrophobic surface of the protein to water or by protein structural changes involving compression of transmembrane helixes since both of these processes should have relatively large changes in free energy associated with them. Deformation of the lipids at the protein boundary appears to be a more likely explanation (Lewis & Engelman, 1983a).

On the basis of the freeze–fracture results discussed above, it can be concluded that the abnormal spectral changes observed after illumination of rhodopsin in recombinant membranes above their characteristic thermal transition temperature are a result of specific interactions between the protein and the lipid in which it is embedded and are not due to protein aggregation.[2] Below the characteristic thermal phase, rhodopsin appears to be separated into dense rhodopsin-rich patches in all of the membrane systems that have been examined [di-14:0-PC, di-16:0-PC, and di-(18:1)$^{t\Delta 9}$-PC], and in each case the spectral transitions after bleaching resemble those of di-14:0-PC rather than the native membrane. On the basis of spin-label studies of recombinant membranes below the characteristic thermal transition temperature (Baldwin & Hubbell, 1985; Davoust et al., 1980), the rhodopsin molecule is likely located in domains of fluid lipid, which is in equilibrium with domains of pure solid lipid. Thus, the unusual spectral behavior in these lipids below T_c after bleaching is not due to rhodopsin being entrapped in a solid matrix.

From the results presented above, it is evident that under three circumstances meta II is not formed in appreciable amounts. First, meta II is not observed above or below the phase transition if the acyl chains are too short, i.e., 14 or fewer carbon atoms in length. In recombinants with acyl chains this short, even the addition of a cis double bond does not restore the normal protein behavior. Second, observable amounts of meta II are not formed in the presence of only *n*-alkyl acyl chains, e.g., those derived from lauric, myristic, and palmitic acids. That this is not simply a chain-length effect can be seen by comparing di-16:0-PC (no meta II formation) with two lipids in which formation of meta II is observed, diphytanoyl-PC, a chain of the same length with four additional side-chain methyl groups, and di-(18:1)$^{c\Delta 9}$-PC, which forms bilayers that are only slightly thicker than those formed by di-16:0-PC due to its cis double bond (Lewis & Engelman, 1983b). The third condition under which the meta II population is suppressed is below the characteristic thermal transition temperature of the recombinant membranes. Meta II formation was not observed below T_c in any of the recombinants tested, including di-(18:1)$^{t\Delta 9}$-PC in which meta II formation *is* observed above T_c.

The observation of normal protein behavior in the diphytanoyl-PC recombinants demonstrates that unsaturation per se is *not* required for the occurrence of the meta I to meta II transition. This rules out a specific interaction role for double bonds and suggests that the major role of unsaturation is a structural one within the bilayer.

In recombinant membranes where meta II formation is not observed at pH 6.5, lowering the pH of the surrounding medium to ≈5.5 apparently permits observable amounts of meta II to be formed. As discussed in the previous paper, this important result is consistent with the generally accepted idea that a proton-dependent meta I/meta II equilibrium exists in the recombinant membranes and an increase in H^+ concentration shifts the equilibrium toward meta II formation. According to this notion, the fact that certain lipid environments result in low populations of meta II simply results from a modulation of meta I/meta II free energies as a result of changes in lipid–protein interaction.

As was discussed in the preceding paper (Baldwin & Hubbell, 1985), an unfavorable lipid–protein interaction (and hence a large lipid–protein interfacial free energy, γ_{pl}) may result in an effective pressure on the rhodopsin molecule. In order to undergo the expansion that occurs during the transition from meta I to meta II, the protein must do work against this pressure, and this work makes a positive contribution to the free energy of meta II relative to that of meta I. The experiments presented here represent an attempt to determine the molecular nature of the unfavorable lipid–protein interaction that might contribute to a positive γ_{pl}.

A possible explanation of the unfavorable protein solvation observed in certain lipid environments is that it results from the roughness (irregularity) of the protein surface, a concept that is suggested by the disordering of the surrounding lipid acyl chains observed by NMR (Deese et al., 1981). If this is the case, the results described here generally can be explained on the basis of the hypothesis that the acyl chains adjacent to the protein are required to assume conformations resulting in bent acyl chains (such as gauche kinks) in order to effectively solvate the irregular surface of the protein. For example, in the case of acyl chains that are shorter (in their normal fluid phase) than the hydrophobic thickness of rho-

[2] However, the individual particles in the freeze–fracture images are larger than individual protein molecules. This is seen in both native and reconstituted membranes and could result from small aggregates of rhodopsin (three to five molecules). Rotational diffusion times measured in the native membranes (Brown, 1972; Cone, 1972) suggest that rhodopsin is monomeric, and therefore, we believe that it is likely that the larger than expected particle size is an artifact of an insufficiently fast freezing rate. In any case, this does not modify our conclusion that the absence of meta II formation in the recombinant membranes is not due to mechanical restraint of the protein resulting from an incompressible environment that would be provided by an extensively aggregated domain.

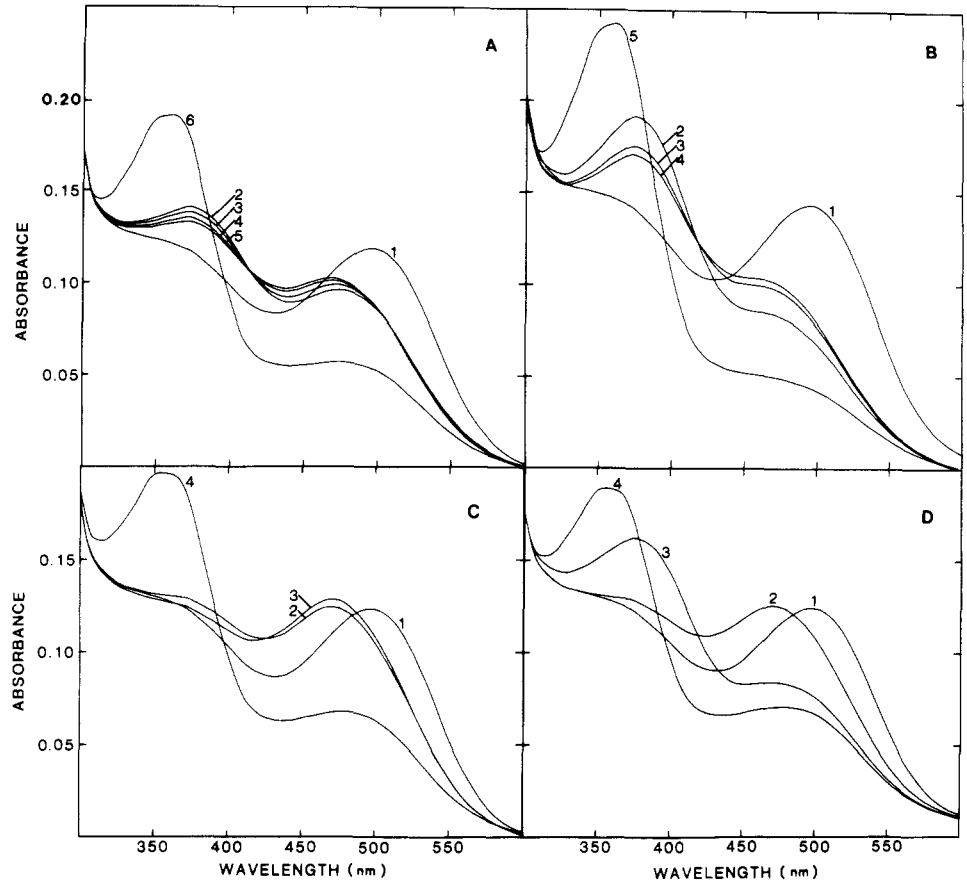

FIGURE 4: Absorption spectra of a suspension of diphytanoyl-PC recombinant membranes in 0.1 M KCl–0.01 M MOPS, pH 6.5. In panels A (15 °C) and B (30 °C), curve 1 was taken before illumination. Curves 2–4 were scanned starting at about 1, 4, and 15 min (7 min for B) after illumination, respectively. In panel A, curve 5 was scanned starting at 30 min after illumination. In panel B, a spectrum taken 15 min after illumination was identical with curve 4. The final curve in panels A and B was scanned in the presence of 0.05 M hydroxylamine added after illumination and shows the amount of rhodopsin plus isorhodopsin remaining in the sample. Panel C shows the effect of a pH increase on the spectral intermediates observed after illumination at 3 °C. Curve 1 was recorded before illumination at pH 6.5, and curve 2 was scanned starting at 1 min after illumination was started. After the recording of curve 2, the pH was raised to ≈7.5, and curve 3 was taken 4 min after illumination. A spectrum identical with curve 3 was observed at 7 min after illumination. Curve 4 is in the presence of 0.05 M hydroxylamine added after the preceding spectra were obtained. Panel D demonstrates the effect of a decrease in pH on the spectral intermediates observed after illumination at 3 °C. Curve 1 was recorded before illumination, and curve 2 was scanned starting 1 min after illumination started. After the recording of curve 2, the pH was lowered to 5.5, and curve 3 was obtained 4 min after illumination. Curve 4 is in the presence of 0.05 M hydroxylamine.

dopsin, the hydrophobic mismatch between lipid and protein can be relieved by reducing the density of the gauche conformations that normally occur in fluid-phase lipid (usually three to four gauche bonds per chain; Nagle, 1973; Marcelja, 1974; Seelig & Niederberger, 1974). Thus, in short acyl chain lipids, very few gauche bonds are present, resulting in unfavorable protein solvation due to lack of conformational flexibility.

The difference between the di-16:0-PC recombinant and the other recombinants that are of similar length but contain additional methyl groups or unsaturation can also be explained on the basis of a need for conformational flexibility in order to effectively solvate rhodopsin. In this case, the energy of a gauche kink relative to a trans configuration is much larger in the *n*-alkyl lipid than for a single bond next to a double bond (Flory, 1969) or in a methylated chain (Lindsey et al., 1979). Of course, for the acyl chains containing cis double bonds, this in itself results in a bend at that point in the chain. Finally, this hypothesis could also explain the results obtained in the recombinants below their T_c, where the gauche kink density should be relatively low and solvation consequently poor.

On the basis of the results presented here, lipids containing fluid-phase acyl chains that are greater than or equal to 16 carbon atoms in length and are not *n*-alkyl are required for an observable meta I to meta II transition near room temperature at neutral pH. Clearly, the large amounts of 22-carbon acyl chains with four, five, or six double bonds found in ROS membranes meet these criteria. However, the long length and high degree of unsaturation of the native acyl chains is not necessary for the transition to occur, and thus, it is likely that they serve some additional purpose(s) in the ROS membrane. In the recombinant systems where the meta I to meta II transition was observed, the quantitative details of the transition were altered compared to native membranes. Thus, it is probable that the long, polyunsaturated acyl chains provide an environment that is fine tuned to produce the optimal ratio and decay rates of meta I and meta II. In addition, the structure of these acyl chains may provide a good "fit" at the protein surface and thus may be responsible for sealing the membranes against leakage of small molecules.

An additional role for these acyl chains is suggested by the presence of both high levels of Ca^{2+} (Liebman, 1974; Szuts & Cone, 1977) and phosphatidylserine (Miljanich, 1978; Fliesler & Anderson, 1983) in the rod outer segments. Bilayers containing PS are known to undergo lateral phase separations under the influence of Ca^{2+} due to binding of the divalent cation to the acidic phospholipids (Jacobson & Papahadjopoulos, 1975). However, it has been demonstrated that no

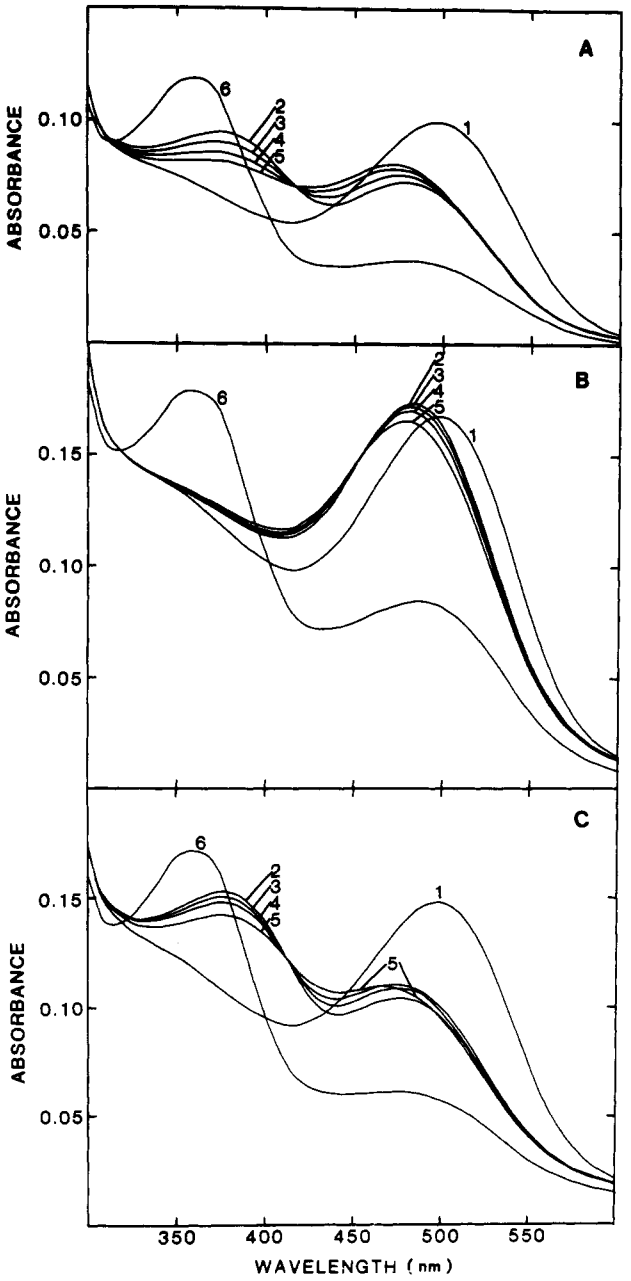

FIGURE 5: Absorption spectra of a suspension of di-(18:1)$^{t\Delta 9}$-PC recombinant membranes in 0.1 M sodium phosphate: (A) pH 6.5, 25 °C; (B) pH 6.5, 5.6 °C; (C) pH 5.0, 5.6 °C. In each panel, curve 1 was obtained before illumination. Curves 2–5 were scanned starting at the following times after illumination: (panel A) 0.5, 1.5, 4.5, and 10.5 min; (panel B) 1.5, 12, 27, and 67 min; (panel C) 0.5, 3.0, 8.5, and 63.5 min. In each panel, curve 6 was scanned in the presence of 0.05 M hydroxylamine added after the preceding spectra were obtained.

complexation with Ca^{2+} of extracted ROS PS occurs at physiological temperatures and that the acyl chains remain disordered in the presence of Ca^{2+} (Sklar et al., 1979). In the rod outer segment, at least 98% of the ROS PS molecules contain polyunsaturated acyl chains of which about 40% contain two polyunsaturated chains (Miljanich et al., 1979). By contrast, experiments on bovine brain PS, which contains a much smaller percentage of polyunsaturated chains, demonstrate that Ca^{2+} produces a complex in which the acyl chains are ordered at physiological temperatures (Sklar et al., 1979). Thus it seems likely that a major role played by the long, polyunsaturated acyl chains in ROS membranes is prevention of a Ca^{2+}-induced lateral phase separation of PS, which could result in abnormal protein behavior. Support for this idea comes from a recent study by de Grip et al. (1983) in which the light-induced photochemistry of recombinant membranes made with either endogenous ROS lipids or bovine spinal cord PS was compared in the presence and absence of Ca^{2+}. The photolytic behavior was not affected by Ca^{2+} in the native lipid recombinants, but an abnormal sequence of photointermediates similar to that discussed here was seen in the presence of Ca^{2+} for the PS recombinants.

Registry No. Di-(14:1)$^{c\Delta 9}$-PC, 56750-90-4; di-14:0-PC, 18194-24-6; di-16:0-PC, 63-89-8; di-(18:1)$^{t\Delta 9}$-PC, 56782-46-8; diphytanoyl-PC, 32448-32-1.

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