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Effect of Partial Delipidation of Purple Membrane on the Photodynamics of Bacteriorhodopsin[†,‡]

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ABSTRACT: The effect of lipid-protein interaction on the photodynamics of bacteriorhodopsin (bR) was investigated by using partially delipidated purple membrane (pm). When pm was incubated with a mild detergent, Tween 20, the two major lipid components of pm, phospholipids and glycolipids, were released in different ways: the amount of phospholipids released was proportional to the logarithm of the incubation time; the release of glycolipids became noticeable after the release of ~2 phospholipids/bR, but soon leveled off at ~50% of the initial content. It was found that the thermal decay of the photocycle intermediate N_{560} was inhibited by the removal of less than 2 phospholipids per bR. This inhibition was partly explained by an increase in the local pH near the membrane surface. More significant changes in the bR photoreactions were observed when >2 phospholipids/bR were removed: (1) the extent of light adaptation became much smaller, and this reduction correlated with the release of glycolipids; (2) N_{560} became difficult to detect; (3) the M_{412} intermediate, which is characterized by a pH-insensitive lifetime, was replaced by a long-lived M-like photoproduct with a pH-sensitive lifetime. The heavy delipidation apparently altered the mechanism by which the deprotonated Schiff base receives a proton. An important conformational change in the protein moiety is suggested to take place during the M_{412} state, this conformational change being inhibited in the rigid lipid environment.

Bacteriorhodopsin (bR),[1] the sole protein in the purple membrane (pm) of *Halobacterium halobium*, functions as a light-driven proton pump. [for reviews, see Stoeckenius et al. (1979), Khorana (1988), Kouyama et al. (1988a), and Rothschild (1988)]. In the living cells and isolated pm fragments, bR exists in two interconvertible isomers, one containing *all-trans*- and the other 13-*cis*-retinal. The equilibrium between them is slowly established in the dark (dark-adapted state). The trans isomer (bR_{570}) accumulates rapidly in the light (light-adapted state), and its cyclic photoreaction drives the translocation of proton from the cytoplasmic side to the outside. At low light intensity, the trans photocycle is approximated by the scheme (Kouyama et al., 1988b; Lozier et

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[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tween 20, poly(oxyethylene) (20) sorbitan monolaurate; DOC, deoxycholic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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al., 1975) $bR_{570} \rightsquigarrow K_{590} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{570}$; here numbers indicate the estimated absorption maxima. The formation of the blue-shifted intermediate M_{412} accompanies deprotonation of the Schiff base of the retinal chromophore. The N_{560} intermediate, which has a slightly blue shifted absorption maximum and slightly less extinction than bR_{570}, is difficult to detect at or below neutral pH, because its lifetime is comparable to or shorter than its rise time. But, when the pH of the cytoplasmic side medium is increased, the thermal decay of N_{560} is inhibited. It is suggested that the retinal chromophore in N_{560} is reprotonated from a group inside the membrane, which later receives a proton from the cytoplasmic side medium (Drachev et al., 1986; Kouyama et al., 1988b, 1989). A recent Raman study of N_{560} has shown that reprotonation of the Schiff base precedes isomerization around the C13–14 double bond (Fodor et al., 1988). To explain a unidirectional movement of proton during the bR photocycle, one needs to postulate that some conformation change in the protein moiety takes place before the transition $M_{412} \rightarrow N_{560}$. Thus, if this conformational change is inhibited in a rigid lipid environment, the last parts of the trans photocycle would be greatly affected.

This paper reports the effect of partial delipidation of pm on the photodynamics of bR. In native pm, bR trimers are arranged in a two-dimensional hexagonal lattice with a unit cell of 62.5 Å (Henderson & Unwin, 1975), and the spaces between the proteins are filled with lipids. The major lipid components, phospholipids (60% of the total lipids) and glycolipids (30%), have highly branched phytanyl hydrocarbon chains and a negatively charged head group (Kushwaha et al., 1977; Kates et al., 1982). It has been recently reported that the most abundant phospholipid is phosphatidylglycerol phosphate in which the terminal phosphate is O-methylated (Fredrickson et al., 1989; Tsujimoto et al., 1989). The major component of glycolipid in pm is sulfated triglycosyl diether (SO_3^--Gal-Man-Glc-diphytanyl ether) (Kates et al., 1982). A large fraction of the lipids of pm can be removed by extraction with a mild detergent like deoxycholate (DOC) (Hwang & Stoeckenius, 1977; Henderson et al., 1982). The delipidation technique by which good crystalline order is retained has been developed (Glaeser et al., 1985), and the three-dimensional structure of delipidated pm has been determined at 6-Å resolution (Tsygannik & Baldwin, 1987); most lipids are released from the boundary lipid layer surrounding the bR trimer, and three pairs of lipid molecules in the central part of the bR trimer are not affected (Glaeser et al., 1985). Few workers have studied spectroscopic properties of delipidated pm (Szundi & Stoeckenius, 1987; Jang & El-Sayed, 1988; Hartsel & Cassim, 1988).

In the present study, we incubated pm with a mild detergent (Tween 20), and by changing the incubation time from a few minutes to 1 month, we prepared the membranes that contained different amounts and different compositions of lipid. Using such samples, we analyzed the interactions between bR and specific lipid components and their effect on the absorption kinetics of bR.

Materials and Methods

Purple membrane was prepared from *H. halobium* JW3 according to the established procedure (Oesterhelt & Stoeckenius, 1974), and its suspension in ~30% sucrose was stored at −80 °C. Before use, pm was washed extensively with distilled water (more than 4 times). The kinetics of delipidation of pm in the presence of Tween 20 were measured as follows: Two milligrams of pm was incubated at 20 °C in 2 mL of 0.04–4% Tween 20 containing 0.1 M NaCl and 50 mM HEPES (pH 8.0). After the incubation for a desired period (a few minutes to 1 month), pm was sedimented by centrifugation at 200000*g* for 5 min (Beckman TL100) and quickly resuspended in 0.1 M NaCl and 50 mM HEPES (pH 8.0). The washing procedure was repeated more than 3 times, and then bR, phosphorus, and hexose in the pellet were quantified. The bR content in native pm was determined by using the molar extinction coefficient 63 000 M^{-1} cm^{-1} at 570 nm (Rehorek & Heyn, 1979). The bR content in delipidated pm was determined from the absorbance at 280 nm, which was assumed to be unaffected by the delipidation. Lipid phosphorus was measured by the molybdate method after ashing (Gerlach & Deuticke, 1963). Hexose content was determined by the phenol–sulfuric acid procedure (Dubois et al., 1956). Extracted lipids were separated by thin-layer chromatography on silica gel H in a mixture of chloroform/methanol/70% acetic acid (85:22.5:14).

For optical measurements, delipidated samples were washed more than 5 times with 0.1 M NaCl and 50 mM HEPES (pH 8.0). In the presence of the detergent, flash-induced absorption changes were complicated by the presence of a slowly decaying component ($\tau \sim 0.2$ s at 20 °C) which had a negative amplitude in the entire wavelength region; it seemed likely that the detergent-containing membrane changed its global structure upon light absorption, causing a large change in the turbidity. When delipidated samples were extensively washed to remove remaining traces of the detergent, the amplitude of the slow component was reduced to a negligible level.

Absorption spectra in the presence of intense actinic light were measured with a cross-illumination spectrophotometer, in which measuring light and actinic light were alternately separated with mechanical choppers at a frequency of ~200 Hz (Kouyama et al., 1988b). Actinic light from a Xe lamp (300 W) was passed through a heat-absorbing water filter, reflected from a cold mirror (400–700 nm), passed through an optical filter (>540 nm), and then focused into a sample cell. Measuring light, provided from a Shimadzu UV350a spectrophotometer, was passed through the sample cell from the opposite direction to the actinic light and reflected onto a photomultiplier tube (Hamamatsu R374, Hamamatsu, Japan). Its photocurrent was amplified under the supervision of gating pulses from the choppers, digitized, and stored in the memory of a personal computer (NEC PC9800/VM2). Millisecond absorption kinetics measurements were performed with a constant-power Xe flash lamp (Nissin Electronic Co., Ltd., Tokyo) providing light pulses of 10-μs width (Kouyama et al., 1988b). Light pulses (>580 nm) at a frequency of 0.01–0.25 Hz irradiated the sample cell at a right angle to measuring light, and each light pulse excited a few percent of the pigment. The amplified photosignal was fed to a digital memory scope (Iwatsu DS6121, Tokyo) operated in a pretrigger mode.

Results

(*1*) *Kinetics of Delipidation of pm.* The native pm used in the present study contained 6–7 mol of phospholipid (12.8 mol of phosphorus) and 3 mol of glycolipid (8.8 mol of hexose)/mol of bR. These lipids were released in a complex manner when pm was incubated at 20 °C with 0.04–4% Tween 20. The result of the delipidation kinetics is summarized in Figure 1. The amount of phospholipid released was approximately proportional to the logarithm of the incubation time (the solid circles in Figure 1a) and of the concentration of Tween 20 (the triangles in Figure 1a). The release of glycolipids was slow in the initial phase of delipidation (Figure 1b). The analyses of extracted lipids by thin-layer chromatography showed that

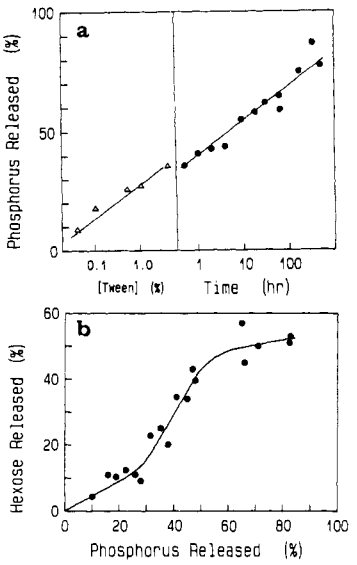

FIGURE 1: Kinetics of delipidation of pm with Tween 20. (a) The amount of lipid phosphorus released is plotted as a function of the incubation time (●) or as a function of the concentration of the detergent (△). In this experiment, pm (1 mg/mL) was incubated at 20 °C with 4% Tween 20 for various periods (●) or with 0.04–4% Tween 20 for 30 min (△). Here the incubation time includes the time period (~10 min) that was required for the sedimentation of pm by centrifugation. (b) The amount of lipid hexose released is plotted as a function of the amount of lipid phosphorus released.

the major component of glycolipid was negligibly released in the initial phase of delipidation. [Carotenoid and cytochrome that remained very slightly in our preparation of native pm were quickly released (~30 min in 0.1% Tween 20); the hexose release in this early stage may be explained by unidentified materials weakly adsorbed to the peripheral part of the membrane.] After 30% of the initial lipid phosphorus was released, the release of glycolipid became significant, but it soon leveled off at ~50% of the initial content. As the result of different delipidation kinetics for phospholipid and glycolipid, the lipid composition in partially delipidated pm was dependent on the extent of delipidation. In the most heavily delipidated sample (1-month incubation with 4% Tween 20), there were ~1.1 mol of phospholipid and 1.4 mol of glycolipid/mol of bR. These figures for the tightly binding lipids are comparable to those found in DOC-treated pm (Glaeser et al. 1985).

(2) Dark/Light Adaptation of bR in Delipidated pm. Spectroscopic properties of delipidated pm were investigated in 0.1 M NaCl and 50 mM HEPES (pH 8.0) at 20 °C. In Figure 2a, the absorption increase at 588 nm due to the light adaptation is plotted as a function of the extent of delipidation. A significant reduction in the extent of light adaptation was seen in the second phase of delipidation during which approximately 1 glycolipid and 1 phospholipid/bR were released. This reduction was explained by a blue shift (by 7–8 nm) of the absorption spectrum in the light-adapted state (a state of the ensemble of bR molecules, which could be a mixture of bR isomers); the absorption maximum (560 ± 1 nm) in the dark-adapted state was little affected by the delipidation, except for the most heavily delipidated pm in which the absorption maximum appeared at 556 nm.

The small extent of light adaptation was not due to a rapid dark adaptation. In fact, the half-time of dark adaptation in heavily delipidated pm was about twice longer than in native pm.

(3) Absorption Kinetics of Delipidated pm. When briefly delipidated pm was irradiated by a 10-μs light flash (>580 nm), the absorption kinetics in the millisecond time region was dependent on the measuring wavelength; that is, a slow component due to the decay of N_{560} was significant at 530 nm but negligible at 410 nm. In Figure 2b, the lifetimes of N_{560} and M_{412} are plotted as a function of the amount of lipid phosphorus released. The lifetime of N_{560} increased exponentially with the increase in the extent of delipidation until 30% of the initial lipid phosphorus (~2 phospholipids/bR) was removed. On the other hand, the decay kinetics of M_{412} scarcely changed (τ ~ 3 ms) throughout the initial phase of delipidation. The inhibition of N_{560} decay in briefly delipidated pm was also

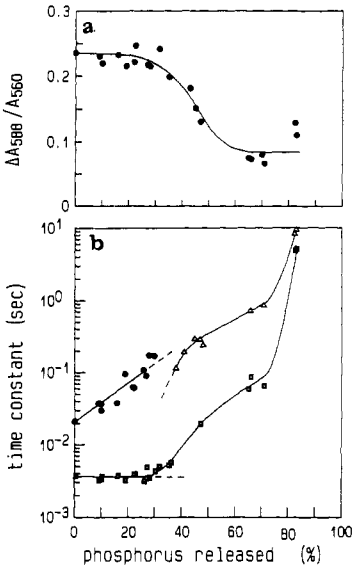

FIGURE 2: (a) Extent of light adaptation vs the extent of delipidation. Membrane suspensions in 0.1 M NaCl and 50 mM HEPES (pH 8.0) were kept in the dark at 20 °C for more than half a day and then light-adapted with orange light (30 mW/cm²; 540–700 nm). The difference absorption spectrum due to the light adaptation had a positive peak at 588 (±1) nm, independent of the extent of delipidation. In the figure, the ratio of the absorption increase at 588 nm to the absorbance at 560 nm in the dark-adapted state is plotted as a function of the amount of lipid phosphorus released. (b) Millisecond absorption kinetics of light-adapted samples. (□) Lifetime of M_{412} or the inverse of the initial rate of the absorption decay at 410 nm observed after a 10-μs light flash (>580 nm). (●) Lifetime of N_{560}, which was calculated from the slow decay component in the absorption transient at 530 nm. (△) Relaxation time of the absorption decay at 410 nm observed after 4 s of illumination with orange light (540–700 nm) at 30 mW/cm²; the time at which the absorbance decayed by a factor of e is plotted.

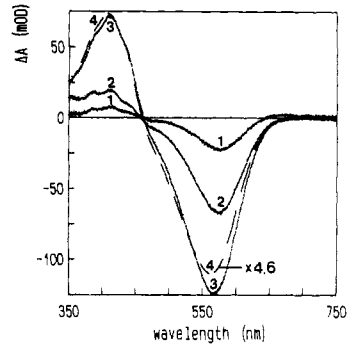

FIGURE 3: Light-induced difference absorption spectra of native and delipidated pm. The extent of delipidation is 0% (trace 1), 28% (trace 2), 45% (trace 3), and 83% (trace 4). (Trace 4 was renormalized so as to have the same amplitude at 410 nm as trace 3.) A light-adapted sample (OD_{570} ~ 0.55) in 0.1 M NaCl and 50 mM HEPES (pH 8.0) at 20 °C was irradiated by continuous orange light (30 mW/cm²; 540–700 nm), and the absorption spectrum recorded in the light was subtracted by the spectrum recorded ~2 min after the light was turned off.

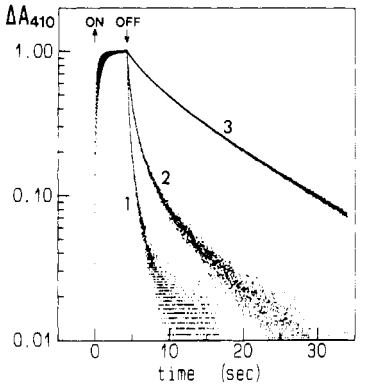

FIGURE 4: Light-induced absorption changes at 410 nm of delipidated pm. The extent of delipidation is 47% (trace 1), 70% (trace 2), and 83% (trace 3). A light-adapted sample ($OD_{570} \sim 0.55$) in 0.1 M NaCl and 50 mM HEPES (pH 8.0) at 20 °C was irradiated by orange light at 30 mW/cm^2 for 4 s every 100 s. As a base line for the calculation of the lifetime, the absorbance observed just before the illumination was used. In native pm or briefly delipidated pm, the absorption decay completed within 20 ms.

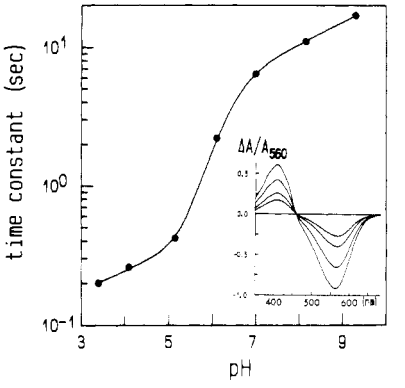

FIGURE 5: pH dependence of the lifetime of the 410-nm-absorbing photoproduct in the most heavily delipidated pm. The lifetime was calculated from the absorption kinetics at 410 nm observed after a 10-μs light pulse. (Insert) Light-induced difference absorption spectra observed at pH 3.5, 5.1, 6.2, and 8.0; the absorption spectrum recorded in the orange light (30 mW/cm^2; 540–700 nm) was subtracted by the spectrum recorded ~2 min after the light was turned off. Solvent condition: 0.1 M NaCl, 50 mM HEPES, at 20 °C.

suggested by the light-induced difference absorption spectra of native and delipidated pm (traces 1 and 2 in Figure 3). When native pm was irradiated by orange light (540–700 nm, 30 mW/cm^2), the depletion of the visible absorption band was accompanied by small absorption increases at 410 and 640 nm, which are due to the accumulation of M_{412} and O_{640} (Lozier et al., 1975). In briefly delipidated pm, the light-induced difference absorption spectrum exhibited a large negative band at 575 nm and a small positive peak at 410 nm (trace 2). This feature is expected when the lifetime of N_{560} is much longer than the lifetime of M_{412}; the difference spectrum between bR_{570} and N_{560} is shown to exhibit a large negative peak at 580 nm and small positive peaks in the near-UV region (Kouyama et al., 1988b).

When more than 30% of the initial lipid phosphorus was removed, the absorption decay kinetics at 410 nm became complex; i.e., there were many exponential components with lifetimes ranging from a few milliseconds to several seconds. The relative amplitudes of slowly decaying components became larger with the increase of the extent of delipidation. Reflecting these slow decay components, the light-induced difference spectrum of heavily delipidated pm exhibited a large positive band at 410 nm (trace 3 in Figure 3). In such samples, the lifetime of N_{560} was difficult to evaluate. Especially when >50% of the initial lipid phosphorus was extracted, the wavelength dependence of the absorption kinetics in the millisecond to second time region became undetectable.

The absorption decay kinetics of heavily delipidated samples were strongly dependent on the duration of actinic light; i.e., slower components in the absorption decay at 410 nm were more emphasized as the duration of illumination was increased. Figure 4 shows the absorption changes observed when delipidated samples were irradiated for 4 s by orange light at 30 mW/cm^2. The relaxation time of the 410-nm absorption decay after 4 s of illumination was much longer (by a factor of >10) than the average lifetime of the 410-nm photoproduct calculated from flash-induced absorption changes; compare the triangles and the open squares in Figure 2. The relative amplitudes of the slowly decaying components became larger as the intensity of actinic light was reduced. Thus, it is unlikely that the cooperativity between neighboring proteins is responsible for the slow components. In the most heavily delipidated sample, on the other hand, the absorption decay at 410 nm was described with a small number of time constants which differed only slightly from each other (trace 3 in Figure 4), and deviation from a single-exponential decay became much smaller at lower light intensity.

(*4*) *pH Dependence of Absorption Kinetics of Delipidated pm.* In native or briefly delipidated pm, the thermal decay of N_{560} was inhibited at high pH, but the lifetime of M_{412} was insensitive to the medium pH. In heavily delipidated pm, on the other hand, the absorption decay kinetics at 410 nm was strongly dependent on the medium pH. Figure 5 shows the pH dependence of the lifetime of the 410-nm-absorbing photoproduct in the most heavily delipidated pm. In this sample, the profile of the light-induced difference spectrum scarcely changed even when the medium pH was made very acidic (pH ~2); neither N_{560} nor O_{640} accumulated in the light. (A small blue shift of the depletion maximum with increasing pH was seen, but its reason is presently unknown.) It appears that the mechanism by which the deprotonated Schiff base receives a proton from the medium is altered by the extraction of a large fraction of lipids from pm. In native pm, the Schiff base receives a proton from a proton donor within the protein, which later receives a proton from the cytoplasmic surface (Kouyama et al., 1988); in heavily delipidated pm, the Schiff base appears to receive a proton from the external medium directly.

Discussion

In has been shown that glycolipids in pm occur exclusively on the external side of the lipid bilayer (Henderson et al., 1978). As judged from the lipid composition of native pm, it is likely that one or two phospholipids occur in the external side and the other four or five on the cytoplasmic side. Thus, the result shown in Figure 1 suggests that the delipidation process of pm is roughly described by the following three phases: In the initial phase of delipidation (<30 min in 4% Tween 20), 2 phospholipids/bR (presumably one phospholipid from each side of the bilayer) and a much smaller number of glycolipids are released. In the second phase (30 min–10 h), the release of one glycolipid is accompanied by the release of one phospholipid. In the third phase (10 h–1 month), 2 phospholipids/bR and a negligible number of glycolipids are released.

These three phases are shown to have their distinct effects on the photodynamics of bR. The removal of a small number of lipids (<2 phospholipids/bR) caused inhibition of the thermal relaxation of N_{560} without changing other parts of the

photocycle. The second phase of delipidation was accompanied by a decrease in the extent of light adaptation. Also, slowly decaying components of M_{412} or long-lived M-like photoproducts appeared, and their amplitudes became larger with increasing extent of delipidation. A significant increase in the half-time of the 410-nm absorption decay was seen at the last step of the third phase. In the most heavily delipidated pm, the absorption decay at 410 nm after weak light was turned off was approximated with a single exponential, the time constant of which could be longer than 10 s at room temperature (above pH 8). These changes will be discussed later in more detail.

Here we consider the multiplicity of lifetime of M_{412}. In native or briefly delipidated pm, the decay kinetics of M_{412} was not described with a single time constant, but the time constants of major decay components were in a narrow time region (between 1.5 and 9 ms at pH 8 and 20 °C). In heavily delipidated pm, on the other hand, decay time constants that were required to fit the absorption decay kinetics at 410 nm ranged from a few milliseconds to several seconds. This divergence explains a significant dependence of the absorption kinetics on the duration of illumination (the triangles and squares in Figure 2), because a slowly decaying component would be more emphasized with increasing duration of illumination. The light-intensity dependence of the absorption kinetics suggested that the cooperativity between neighboring proteins or the multiphoton excitation had a minor effect. It should be noted that the multiplicity of lifetime became very significant when the major component of glycolipid was released noticeably. Among many possible causes of the multiplicity of lifetime, the one that we should consider is heterogeneity in the lipid environment. It has been reported that the main glycolipid in pm, sulfated triglycosyl diether, is not found in other parts of the plasma membrane in halobacteria (Kates et al., 1982). It is likely that the binding constant of this lipid component to the bR molecule is considerably high. If this is the case, the lipid molecules cannot move around very much during the photocycle and, especially in heavily delipidated pm, the condition for a homogeneous lipid environment cannot be attained.

(*1*) *Decay Kinetics of N_{560} in Briefly Delipidated pm.* The inhibition of the thermal relaxation of N_{560} in briefly delipidated pm can be explained by the increase of the local pH near the membrane surface. In an aqueous suspension of native pm, the local pH near the membrane surface is much lower than the bulk pH, because the major lipid components of pm are negatively charged at neutral pH (Szundi & Stoeckenius, 1987). Thus, the removal of these negatively charged lipids is expected to have the same effect as the increase of the medium pH. It has been shown that, in alkaline suspensions of native pm, the lifetime of N_{560} increases exponentially with the increase of the medium pH (Kouyama et al., 1988b). A similar pH dependence of the lifetime of N_{560} was also seen in a briefly delipidated sample; i.e., the lifetime increased by a factor of 8–10 as the medium pH was increased by 1 pH unit. It is calculated from the data in Figure 2b (solid circles) that the delipidation of 1 phospholipid/bR has the same effect as the increase of the medium pH by 0.4 pH unit (in 0.1 M NaCl). This result agrees qualitatively with the previous observation that the removal of 75% of the initial lipid phosphorus with CHAPS lowered the pK value of the purple-to-blue transition by 2 pH units (in 0.1 mM $CaCl_2$) (Szundi & Stoeckenius, 1987). When the salt concentration used in the present study was taken into account, however, the observed change in the lifetime of N_{560} seems to be larger than expected from the effect of charge removal (Szundi & Stoeckenius, 1989). Presently we cannot exclude other factors such as the rigidity of the lipid environment, which may also affect the lifetime of N_{560}.

Drachev et al. (1986) have found that the lifetime of N_{560}, which they called P, becomes long in the presence of a low concentration (0.05%) of Triton X-100. Probably this phenomenon is also due to the release of a small number of lipids, because the lattice structure of pm has been shown to shrink upon addition of a low concentration of Triton X-100 (Furuno, 1983). Using other detergents (CHAPS, octyl glucoside, and so on), we observed the same phenomenon just after addition of the detergent. It is suggested that the first action of any mild detergent on pm is to pull out lipids from the membrane. However, it should be mentioned that, unlike other detergents, Tween 20 scarcely affected the absorption maximum of the dark-adapted state of pm.

(*2*) *Photodynamics of bR in Heavily Delipidated pm.* The spectroscopic properties of heavily delipidated pm resemble those of a dried film of pm, which also exhibits a very long relaxation time of M_{412} and a small extent of light adaptation (Korenstein et al., 1977; Kouyama et al., 1985). It has been shown that the unit cell dimension of the hexagonal lattice decreases from 62.5 to 61.4 Å when native pm is dried and that this reduction is entirely due to the shrinkage in the area of lipid (Zaccai, 1987). It is expected that any thermal reaction that is accompanied by a large conformation change in the protein moiety is inhibited in a rigid lipid environment. Thus, the very slow relaxation of M_{412} in delipidated pm or in a dried sample is in harmony with the current scheme of the bR photocycle in which an important conformation change in the protein moiety is assumed to take place before the transition $M_{412} \rightarrow N_{560}$ (Fodor et al., 1989). In contrast, the rise time of M_{412} has been shown to be only slightly affected by the delipidation (Jang & El-Sayed, 1989). (Their observation of an apparently lower quantum efficiency of M_{412} formation in delipidated pm can be explained by a smaller extent of light adaptation.) Conformation changes which may take place at the fast steps of the photocycle (before the formation of M_{412}) are suggested to be restricted around the retinal chromophore. A recent FTIR study has shown that small changes of the protein backbone take place with a short delay (0.2 ms) after the formation of M_{412} and that these conformational changes are inhibited in the absence of liquid water (Braiman et al., 1987; Mantele et al., 1982).

From absorption dichroism measurements of native and glutaraldehyde-treated pm, Ahl and Cone (1984) suggested that the bR molecules in native pm undergo large rotations (>10–20°) within the membrane during the photocycle. We failed to confirm their result (unpublished data). But their observation of a dramatic increase in the lifetime of M_{412} upon glutaraldehyde fixation of the membrane is worth noting. Treatment of pm with $LaCl_3$ also slows down the decay of M_{412}, though its formation rate is only slightly affected (Drachev et al., 1984). They suggested that the lanthanum effect is associated not only with altered surface charge of the membrane but also with modification of the protein. Because its effect is reversed by EDTA, it is very likely that a lanthanum ion acts as a cross-linker between negatively charged groups in the protein. Thus, the effects of delipidation, dehydration, glutaraldehyde fixation, and lanthanum binding can be discussed on the same ground; i.e., any treatment that inhibits large conformational changes in the protein moiety would affect the decay kinetics of M_{412}. It has been reported that the proton pumping activity of bR is lost in the presence

of $LaCl_3$ (Drachev et al., 1984). Thus, the conformational changes under discussion seem to be indispensable for the active proton translocation.

(3) Photoconversion between the 13-Cis and All-Trans Isomers. It has been shown that when a dried film of pm is irradiated at the red edge of the absorption spectrum, the dark adaptation is accelerated (Kouyama et al., 1985). We found the same red-light effect in some preparations of delipidated pm, e.g., the Tween-treated pm in which 60–70% of the initial lipid phosphorus was removed or the DOC-treated pm prepared according to the method of Glaeser et al. (1985). In such samples, the small extent of light adaptation is readily explained by a branching reaction from the trans cycle to the cis cycle, which can be significant when the transition $M_{412} \rightarrow N_{560}$ is blocked in a rigid lipid environment.

In the most heavily delipidated pm prepared with Tween 20, however, the red-light effect was not significant. When this sample was exposed to strong light (30 mW/cm^2), the visible absorption band (λ_{max} = 562 nm) was almost completely bleached and the light-induced difference spectrum had a negative band at 562 nm (trace 4 in Figure 3). This result excludes the possibility that the photoconversion from the 13-cis (bR_{550}) to the trans isomer (br_{570}) is completely inhibited, unless excitation of bR_{550} also produces an M-like photoproduct. [Actually, the absorption change at 410 nm induced by weak light became larger (1.5 times) in the light-adapted state than in the dark-adapted state.] To explain the position of the negative band, however, we have to assume that the 410-nm-absorbing photoproduct is generated from a ground state with an absorption maximum near 562 nm. It is possible that, in the most heavily delipidated pm, the absorption spectra of the 13-cis and all-trans isomers are affected differently, and that the absorption change due to light adaptation is small even though the full conversion to the all-trans isomer has occurred. Such a case has been observed in nitrated pm (Scherrer & Stoeckenius, 1985).

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