Photoreaction of Bacteriorhodopsin at High pH: Origins of the Slow Decay Component of M†

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ABSTRACT: The absorption spectrum of light-adapted purple membrane in 3 M KCl is dependent on temperature even in the room temperature region. Temperature-induced difference spectra at various pH values suggested that the trans isomer of bacteriorhodopsin, bR_{570}, is in thermal and/or photodynamic equilibrium with several different conformers. The major second conformer occurring at neutral pH had the same spectroscopic properties as the 13-cis isomer, and its content at 35 °C was estimated to be more than 20%. Heterogeneity in the protein conformation became more significant above pH 8, where temperature-induced difference spectra exhibited a negative peak at 580 nm and a positive peak at 296 nm. This absorption change is very similar to that observed upon the formation of the N intermediate, suggesting that an N-like conformer occurs at high pH and temperature. A significant temperature dependence was also seen in the M decay kinetics at high pH, which were described by two decay components; i.e., the fast decaying M (M^f) was predominant at low temperature, but the amplitude of the slow component (M^s) increased with increasing temperature. It is suggested that M^s is generated upon excitation of the N-like conformer, in which the residue (Asp-96) usually acting as a proton donor to the Schiff base is deprotonated. The N-like conformer could be N itself, because M^s was enhanced when N was accumulated by background light. A strong correlation between the amplitude of M^s and the concentration of N was also revealed by the accumulation kinetics of M^f, M^s, and N after the onset of continuous actinic light. This correlation is explained readily by the photoreaction of N leading to M^s. These results do not necessarily exclude other origins of M^s, however. To explain a large amplitude of M^s at extremely high pH (pH > 10), we may have to assume another ground state in which Asp-96 is deprotonated and the retinal chromophore is in the trans configuration. Also, a cooperative mechanism of the production of M^s cannot be neglected at high excitation intensity. But the back-reaction N → M, which had been proposed to explain M^s, was shown to be insignificant at moderately high pH.

Bacteriorhodopsin, a membrane protein found in *Halobacterium halobium*, functions as a light-energy-converting proton pump. Its polypeptide chain is folded into a conformation consisting of seven transmembrane α-helices, and the retinal chromophore is linked by a protonated Schiff base to Lys-216 which is buried within the interior of the protein (Henderson et al., 1990). When the pigment containing *all-trans*-retinal (bR_{570})[1] absorbs light, it undergoes a cyclic photoreaction that drives proton translocation across the membrane. This trans photocycle is approximated by the following scheme: $bR_{570} \rightsquigarrow K_{590} \rightarrow L_{550} \rightarrow M_{410} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{570}$ (Lozier et al., 1975; Kouyama et al., 1988; Fodor et al., 1988). The initial step involves a trans-to-cis isomerization of the chromophore at the C13–C14 double bond. In the L → M transition, the Schiff base deprotonates and Asp-85 mediates proton transfer from the Schiff base to the extracellular membrane surface. In the M → N transition, the chromophore is reprotonated from Asp-96, which in turn receives a proton from the cytoplasmic side. The chromophore reisomerizes to the all-trans configuration and the protein relaxes to the initial state.

A protonated aspartic acid (Asp-96) positioned in the proton-uptake pathway is an important feature of the protein architecture of bR. Its presence grants that the deprotonated Schiff base in the M state receives a proton from the cytoplasmic side with a high efficiency. Substitution of Asp-96 with a nonprotonatable amino acid causes a significant reduction in the decay rate of M at alkaline pH (Otto et al., 1989; Stern et al., 1989; Holz et al., 1989; Miller & Oesterhelt, 1990). Such bR mutants are defective in generating a large pH gradient, though they show a low pumping activity at low pH (Mogi et al., 1988; Marinetti et al., 1989; Butt et al., 1989). In wild-type bR, the M → N transition is rapid irrespective of the medium pH and, instead, the decay of N is inhibited at high pH. Unlike M, N can be hit back by a second photon without any destructive effect on the proton translocation (Ormos et al., 1978; Butt et al., 1989; Kouyama & Nasuda-Kouyama, 1989; Subramaniam et al., 1991). Thus, the long lifetime of N at high pH does not necessarily lead to a significant reduction in the proton pumping power. It has been shown that wild-type bR exhibits a high pumping activity between 3.5 and 9 and it can generate a pH gradient as large as 4 pH units (Kouyama & Nasuda-Kouyama, 1989; Varo et al., 1989; Liu, 1990).

It is believed that the pK_a value of Asp-96 is unusually high (Braiman et al., 1988; Gerwert et al., 1989; Engelhard et al., 1990). But its exact value has not been determined. Undoubtedly, the protein conformation with a protonated aspartic acid is energetically unfavorable at high pH. Because Asp-96 is depronated in the N state, one may argue that the back-reaction $bR_{570} \rightarrow O_{640} \rightarrow N_{560}$ is no longer neglected at high

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[1] Abbreviations: bR, bacteriorhodopsin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid.

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pH. We have previously suggested that the biphasic decay of M at high pH comes from a thermal equilibrium between bR_{570} and N; i.e., the fast component (M^f) was attributed to the M → N transition and the slow component (M^s) was attributed to the decay of a long-lived M-type product of N (Kouyama et al., 1988). Later, our suggestion was disputed by Ames et al. (1989), who found no difference in the resonance Raman spectrum for the two M forms. More recently, however, Tokaji and Dancshazy (1991) found that the relative amplitude of M^s increases with increasing intensity of excitation light. Their observation reminds us of the earlier suggestion that a cooperative interaction of bR molecules cannot be neglected as long as purple membrane, a two-dimensional crystal of bR, is investigated (Ohno et al., 1981). Because the Raman data were obtained with an intense pumping beam, it is not obvious how the result of Ames et al. is correlated with the biphasic decay of M observed at low excitation intensity. To prove or disprove the back-reaction $bR_{570} \rightarrow O \rightarrow N$, one needs more careful analyses.

In the present study, we investigated the absorption spectrum of light-adapted purple membrane at various temperatures and pH values. It was found that, at high pH, a second conformer with the same spectroscopic property as the N intermediate appeared when the temperature was increased. The temperature-induced absorption change correlated with the appearance of M^s. These results suggest that M^s is generated upon excitation of an N-like conformer. To understand how this second conformer is related to the N intermediate, we further investigated the accumulation kinetics of M^f, M^s, and N after the onset of continuous actinic light as well as the M decay kinetics in the presence of background light. The results obtained are consistent with the previous proposal of the photoreaction of N leading to bR_{570} via a long-lived M-type product. At the same time, we have noticed that our spectroscopic data do not necessarily exclude some of other schemes that were proposed to explain M^s. We shall discuss which scheme should be adopted for better description of the proton pumping activity of bR.

METHODS AND MATERIALS

Cells of *H. halobium* strain JW3 were grown on a complex medium consisting of a basal salt solution supplemented with amino acids (Engelman & Zaccai, 1980). Purple membrane fragments were isolated from washed cells according to the established procedure (Oesterhelt & Stoeckenius, 1974).

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., 1988). Briefly, actinic light from an Xe lamp was passed through a heat-absorbing water filter, reflected from a cold mirror (400–700 nm), passed through optical filters, and then focused into a sample cell. Measuring light, provided from a Shimadzu spectrophotometer UV350a, was passed through the sample cell from the opposite direction to the actinic light and reflected onto a photomultiplier tube. Its photocurrent was sent to a logarithmic amplifier and then to an electric circuit involving sample/hold amplifiers, the output of which was proportional to the difference in absorbance of the sample and reference cell. Subsecond absorption kinetics were measured with a mechanical shutter positioned in the optical path of the actinic light. In measurements of millisecond absorption kinetics, a constant-power Xe-flash lamp and, if necessary, a tungsten lamp for background illumination were attached to the spectrophotometer, in which the chopper positions were fixed and the photomultiplier tube was protected by a combination of interference filters; light pulses at a frequency of 0.1–1 Hz irradiated the sample cell at a right angle to the measuring light; each light pulse had a width of 10 μs.

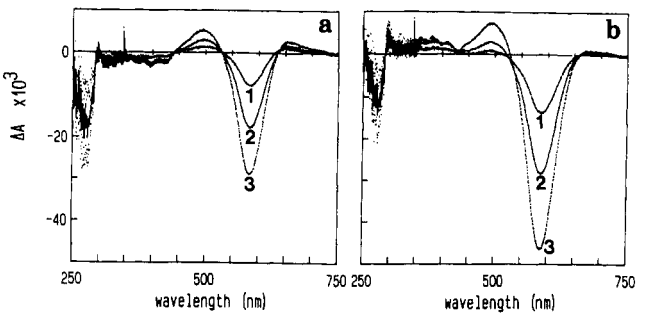

FIGURE 1: Temperature-induced difference spectra of light-adapted purple membrane at pH 7.4 (a) and pH 9.4 (b). A concentrated purple membrane suspension was added to 1.3 mL of 3 M KCl containing 10 mM HEPES (pH 7.4) or 20 mM bicarbonate (pH 9.4) in a 1-cm quartz cuvette, which was then covered tightly with a rubber cap. Absorption spectra were measured at 5 (base line), 15, 25, and 35 °C (traces 1–3). Absorbance at 570 nm was ~0.42 at 15 °C. At each temperature, the suspension was irradiated by green light (500–570 nm) at an intensity of 2.5 mW/cm² for ~10 min, and the wavelength scan from 750 nm to 250 nm at 400 nm/min was started with a short delay (a few seconds at high temperature) after the illumination. Temperature was first increased from 5 °C to 35 °C and then decreased to 5 °C, and two data sets obtained at each temperature were combined for calculation of the difference spectra. The peak at 348 nm is artificial.

RESULTS

(1) *Temperature Dependence of the Absorption Spectrum of Light-Adapted Purple Membrane.* The absorption spectrum of light-adapted purple membrane in 3 M KCl was investigated at pH 6–10 and at 5–35 °C. Figure 1 shows examples of temperature-induced difference spectra. At pH 6–8, the temperature increase was accompanied by large absorption decreases at 585 nm and 280 nm and small absorption increases at 650 nm and 490 nm (Figure 1a). Above pH 8, the 650-nm band in the difference spectra diminished; instead, a positive band at 296 nm became noticeable, and the negative band at 585 nm became more significant (Figure 1b). It is obvious that a temperature-induced expansion of the pm suspension (~0.04%/°C) should be taken into account for correct interpretation of these data. For instance, the negative band at 280 nm would be depressed by 20–40% after correction for the volume expansion. With respect to the negative band at 585 nm, however, the absorption decrease observed (0.3–0.5% decrease/°C) is much larger than expected from the volume expansion.

To explain the spectral changes observed, we need to abandon the widely accepted assumption that the bR conformation is homogeneous in light-adapted purple membrane. Besides the major conformer absorbing maximally at 570 nm (bR_{570}), several conformers with distinct absorption spectra are suggested to occur at high temperature. The 650-nm positive band seen in Figure 1a is indicative of a conformer with a red-shifted absorption maximum. Like O_{640}, this conformer is relatively more stable at lower pH. On the other hand, the large negative band at 585 nm suggests the occurrence of conformers with blue-shifted absorption maxima.

Figure 2 shows difference spectra associated with the transitions of bR_{570} into the well-established bR states. It can be seen that the temperature-induced difference spectra at neutral pH are similar to the difference spectrum associated

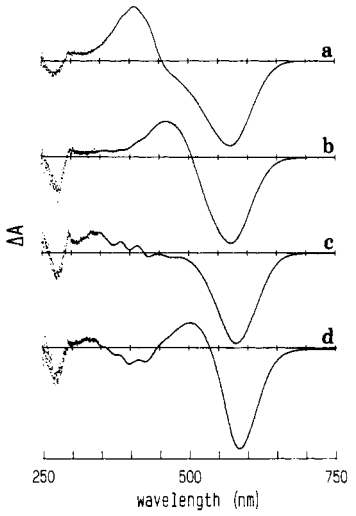

FIGURE 2: Difference spectra associated with the transitions of bR_{570} into the M intermediate (a), the alkaline form bR_{480} (b), the N intermediate (c), and the 13-cis isomer bR_{555} (d). These spectra were obtained by the following procedures. (a) Light-adapted purple membrane in 200 mM guanidine hydrochloride (pH 9.5, 20 °C) was irradiated by orange light (540–700 nm, 30 mW/cm²), a long-lived M-type photoproduct ($\tau \sim 2$ s) being accumulated. (b) Light-adapted purple membrane in 50% dimethyl sulfoxide (+50 mM KCl) was alkalized (pH 6.6 → 8.2) so that purple-to-red transition of bR was partially induced. (c) Light-adapted purple membrane in a 20% polyacrylamide gel at pH 9.4 (+1 M KCl, 10 °C) was irradiated by weak actinic light (540–700 nm, 2 mW/cm²) so that only the N intermediate accumulated significantly. (d) Purple membrane in 3 M KCl at pH 9.3 was kept in the dark for 1 day.

with the dark adaptation (see Figures 1a and 2d). On the assumption[2] that the absorption depletion at 585 nm in Figure 1a is due to the conversion from the trans isomer to the 13-cis isomer (bR_{555}), it is calculated that, at 35 °C, more than 20% of the total amount of bR exists as the 13-cis isomer. This large amount of the 13-cis isomer is not explained by rapid dark adaptation. At 35 °C, the half-time of dark adaptation was 21 min at pH 7.4 in 3 M KCl (80 min at pH 9.4), and maximally 3% of bR molecules was calculated to be converted into the 13-cis bR isomer during the recording period of each absorption spectrum (~1 min). Also, it is unlikely that the light adaptation was incomplete at the light intensity used (2.5 mW/cm²), because essentially the same result was obtained when the sample was light-adapted by stronger light (10 mW/cm²). [At the high light intensity, however, a small amount (~0.5%) of bR molecules was converted into an unidentified product ($\lambda_{max} \sim 400$ nm) during one cycle of the measurement.] One possible explanation of Figure 1a is that the branching reaction from the trans to the cis cycle becomes significant at high temperature, as has been shown in monomeric bR or dehydrated purple membrane (Casadio & Stoeckenius, 1980; Kouyama et al., 1985).

The temperature-induced difference spectra observed at high pH are not completely explained by the conversion of bR_{570} into bR_{555} (or a bR_{555}-like species). Especially trace 1 in Figure 1b is rather similar in profile to the difference spectrum associated with the transition $bR_{570} \rightarrow N$ (Figure 2c). An additional conformer, which we call the N-like conformer, is suggested to occur at high pH. It is worth noting that the $bR_{570} \rightarrow N$ transition is accompanied by a significant absorption increase at 296 nm. The same absorption increase was induced when the temperature was increased (Figure 1b). The positive peak at 296 nm was not seen in the difference spectrum associated with dark adaptation, which did not change its profile between pH 4 and 10. On the assumption that the N-like conformer has the same absorption spectrum as N, it is calculated from the amplitude of the 296-nm band in Figure 1b and the reported absorption spectrum of N that more than 20% of bR molecules are converted into the N-like conformer at 35 °C. [The 490 nm band in Figure 1b suggested that a non-negligible amount of bR_{555} (or a bR_{555}-like species) also appeared at high pH and temperature.]

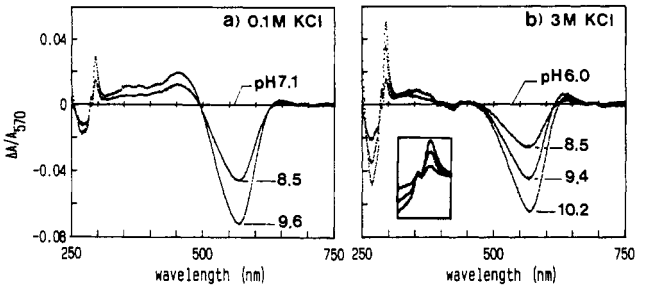

FIGURE 3: Alkali-induced difference spectra of light-adapted purple membrane in 0.1 M KCl (a) and 3 M KCl (b) at 23 °C. Insert in (b): difference spectra between 270 and 310 nm. The data shown were obtained by adding 0.1 N NaOH to fleshly prepared samples ($OD_{570} \sim 0.44$).

(2) *pH Dependence of the Absorption Spectrum of Purple Membrane.* Figure 3 shows alkali-induced difference spectra of light-adapted purple membrane at 23 °C. Alkalization at low ionic strength caused a significant absorption increase at 460 nm. This change is characteristic of formation of a species absorbing maximally at 480 nm (bR_{480}) (compare with Figure 2b). In 3 M KCl, on the other hand, formation of bR_{480} was not noticeable. The present observation is consistent with the previous result that formation of bR_{480} in reconstituted bR vesicles is inhibited by a high concentration of chloride ion (Nasuda-Kouyama et al., 1990).

It can be seen in Figure 3b that the 296-nm band is accompanied by a small shoulder at 287 nm. This shoulder developed above pH 8, and its amplitude changed only slightly between pH 8.5 and 10. A different pH dependence is seen for the 296-nm absorption change, the amplitude of which increased monotonously with increasing pH. As suggested by other workers (Balashov et al., 1991; Scherrer & Stoeckenius, 1984), two things seem to occur in the alkaline pH region; one is deprotonation of a tyrosine residue(s) which is(are) accompanied by an absorption increase at 300 nm; the other is the red shift of the absorption band of tryptophan, causing the doublet peak at 296 nm and 287 nm in the difference spectrum. This red shift has been suggested to be induced by the appearance of a negative charge in the neighborhood of tryptophan (Maeda et al., 1986). The doublet peak at 296 nm and 287 nm was also seen in the difference spectrum associated with formation of N. Because Asp-96 is protonated in bR_{570} and deprotonated in N, the protonation state of Asp-96 could be one of the major factors affecting the absorption spectrum in the UV region.

It should be mentioned that the alkali-induced absorption changes were not completely reversible. Hysteresis was observed especially in the absorption spectrum at the long wavelength. When the pH titration was repeated with the

[2] Scherrer et al. (1988) previously observed temperature-induced absorption changes in dark-adapted purple membrane. Because they found no significant change in the retinal isomer ratio below 40 °C, they attributed the observed absorption changes to conformational changes in the protein moiety. Unlike their difference spectra, the traces in Figure 1a exhibit fine structures at 350–450 nm. This feature is probably due to a blue shift of the β absorption band of retinal, and it is indicative of alternation in the retinal configuration. Presently it is not obvious how their result is correlated with our result.

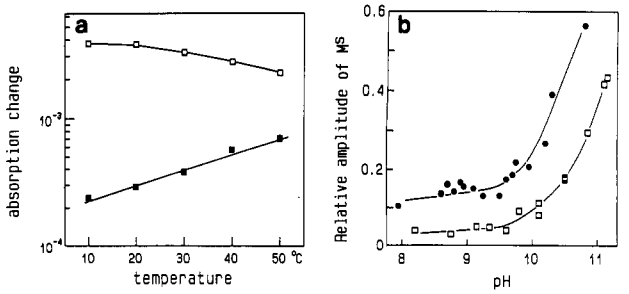

FIGURE 4: (a) Temperature dependence of the amplitude of the slowly decaying component of M (M^s, ■). Open squares show the peak amplitude of flash-induced absorption change at 424 nm. (b) pH dependence of the relative amplitude of M^s at 10 °C (□) and 30 °C (●). Solvent condition: 3 M KCl, 20 mM bicarbonate. Each light pulse (540–620 nm) excited ~2% of bR molecules.

same sample, the positive band at 640 nm in Figure 3b became more significant and, instead, the negative band at 570 nm became less significant. One possible explanation of the observed hysteresis is an irreversible redistribution of lipid molecules in purple membrane, which may cause a shift in the equilibrium among bR_{570}, bR_{555}, and the N-like conformer.

(3) *Temperature and pH Dependences of the M Decay Kinetics.* It has been known that the decay of M is biphasic at high pH; the fast decay component has a pH-insensitive lifetime (~1 ms at room temperature), whereas the lifetime of the slow component increases exponentially with increasing pH (Ohno et al., 1981; Kouyama et al., 1988; Otto et al., 1989). In order to understand how the biphasic decay of M is correlated with the appearance of the N-like conformer, we investigated the millisecond absorption kinetics at various temperatures and pH values. In the following experiments, the decay kinetics of M were measured at 424 nm; the decay constant of N was determined from the slowest decay component at 590 nm, where the difference spectrum between bR_{570} and N has a large amplitude [Figure 2c; also see Kouyama et al. (1988)]. [For the decay constant of N, a consistent result was obtained when the absorption change at 350 nm was investigated.] At pH 8–10, the decay time constant of N was much longer (3–5 times) than that of the slow decay component of M (M^s).

Figure 4a shows the temperature dependence of the decay kinetics of M at pH 9.5. At low temperature, the slow decay component (M^s) was negligible (6–7%). With increasing temperature, the amplitude of M^s increased significantly. This temperature dependence suggests that M^s is generated upon excitation of the N-like conformer, the concentration of which increases with increasing temperature. It can be seen in Figure 4a that the peak amplitude of absorption change at 424 nm decreased slightly. This decrease is explained partly by the appearance of the 13-cis isomer (bR_{555}), the photoreaction of which has been shown to be accompanied by little absorption change at 424 nm (Hofrichter et al., 1989).

In Figure 4b, the relative amplitude of M^s is plotted as a function of pH. The amplitude of M^s was approximately constant at pH 8–10, and it increased sharply above pH 10. A conspicuous difference in the pH dependence below and above pH 10 suggests that the phenomenon observed at very high pH is fundamentally different from the one observed when the temperature was increased at pH 8–10. Although the prominent enhancement of M^s by alkalization is presumably caused by deprotonation of some important residue (e.g., Asp-96) in the ground state, it is not obvious how the protein conformation at very high pH is related to the N-like conformer occurring at pH 8–10. Unfortunately, protein denaturation and/or aggregation was often induced above pH 10, and this

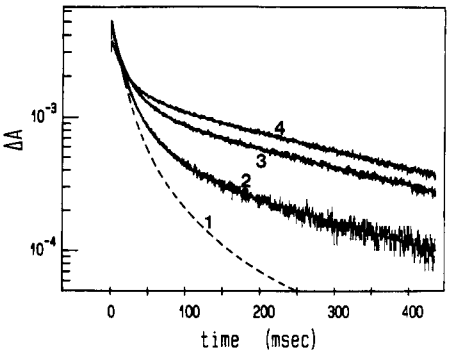

FIGURE 5: Flash-induced absorption changes at 424 nm of light-adapted purple membrane in the absence (traces 1 and 2) and presence (traces 3 and 4) of background light. The intensity of background light (540–620 nm) was 0.55 (trace 3) or 3 mW/cm^2 (trace 4). Traces 2–4 were obtained at the same intensity of excitation light (540–620 nm). Trace 1 was obtained at much lower excitation intensity; this trace is renormalized (by a factor of ~10) so as to have the same peak amplitude as trace 2. A 20% polyacrylamide gel containing purple membrane (width ~1 mm; OD_{570} ~ 0.35) was soaked in 3 M KCl and 10 mM bicarbonate at pH 9.2 and was oriented to make an angle of 45° with the measuring or excitation light beam. Temperature: 10 °C.

made it difficult to quantitatively analyze what occurred at very high pH.

(4) *Effect of Background Light on the M Decay Kinetics.* At pH 8–10 and at low temperature, the millisecond absorption kinetics were strongly affected by background light. Its first effect appeared in the decay rate of N, which increased with increasing intensity of background light. This is explained well by a light-initiated reaction from N to bR_{570}, the quantum efficiency of which was previously estimated to be larger than 0.6 (Kouyama & Nasuda-Kouyama, 1989). Another remarkable effect of background light was seen in the decay kinetics of M. In Figure 5, traces 3 and 4 were obtained under background illumination at different light intensities (0.55–3 mW/cm^2; 540–620 nm). The amplitude of M^s increased with increasing intensity of background light, whereas the peak amplitude decreased. Under the solvent condition used, the N intermediate had a lifetime of 980 ms (in the absence of background light), and only this intermediate was accumulated noticeably by weak background light. The simplest explanation of the observed phenomenon is that M^s is generated upon excitation of N. It is important to note that, at low intensities of background light, the increase in the amplitude of M^s was approximately the same magnitude as the decrease in the peak amplitude. This result imposes the following restriction: M^s is formed so slowly that its contribution to the peak amplitude is negligible. It is suggested from the comparison of the traces in Figure 5 that the rise rate of M^s is comparable to but slightly faster than the decay rate of M^f.

To observe the significant background-light effect, one needs to select the solvent condition carefully. The best condition is at pH 9.0–9.5, in 3 M KCl, and at or below 10 °C. At high temperature, the amplitude of M^s was already large in the absence of background light, and thus the background-light effect on the M decay kinetics was not significant. With a similar reason, no significant background-light effect was detected at very high pH (pH > 10.5). Recently, Bitting et al. (1990) reported that the relative amplitude of M^s rather decreased with increasing intensity of background light. This is presumably because their experimental result was obtained at very high pH (pH 10.5).

In this kind of experiment, it is also important to use weak excitation light. The relative amplitude of M^s is dependent

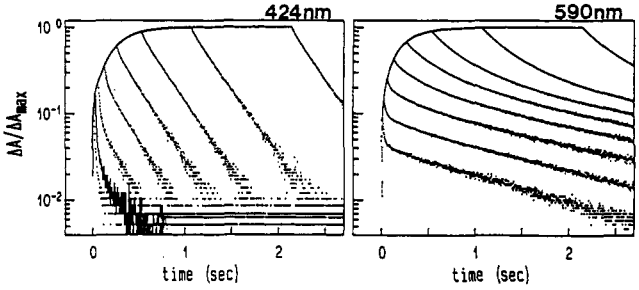

FIGURE 6: Light-induced absorption changes at 424 nm and 590 nm of light-adapted purple membrane (OD_{570} = 0.28) in 3 M KCl and 10 mM bicarbonate at pH 9.3 and 10 °C. The actinic light (540–700 nm) at 30 mW/cm² was turned on at time zero and turned off with various delays. For recording of each trace, the illumination was repeated 10–20 times with a time interval of 10 s. Absorption changes are presented in a logarithmic scale, and the absorbance observed just before the light was turned on is used as a baseline. The ΔA_{max} values were 0.045 at 424 nm and 0.132 at 590 nm.

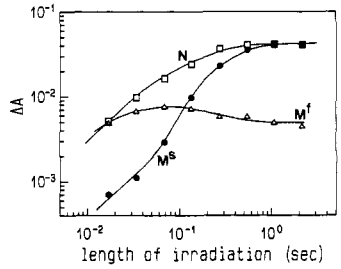

FIGURE 7: Accumulation kinetics of M_f, M_s, and N after the onset of continuous light, deduced from the data as shown in Figure 6. The amounts of M^f and M^s were calculated from double-exponential analyses of the 424-nm absorption decay kinetics, and the amount of N was calculated from the amplitude of the slowest component in the 590-nm absorption decay kinetics.

on the intensity of excitation light. Apparently the increase in the excitation intensity has the same effect as the increase in the intensity of background light (compare traces 1 and 2 in Figure 5). Because the width (10 μs) of each light pulse is much shorter than the rise time of N, the dependence of the M decay kinetics on the excitation intensity is not explained by the photoreaction of N. A cooperative interaction between bR molecules may not be neglected at high excitation intensity, and this effect will be discussed later in more detail.

(5) *Accumulation Kinetics of M^s in Continuous Actinic Light.* To elucidate whether an M-type product is generated upon excitation of N, we further investigated the accumulation kinetics of M^s and N after the onset of continuous actinic light (Figure 6). In this experiment, light-adapted purple membrane was irradiated by continuous orange light (540–700 nm, 30 mW/cm²) for different periods. When the length of irradiation was short (<20 ms), the decay kinetics at 590 nm were approximated by two time constants, 10 ms and 1400 ms. At 424 nm, the 10-ms component (M^f) was dominant; the 1400-ms decay component was not detected and, instead, another decay component with a shorter time constant was seen. These decay kinetics were essentially the same as obtained with the 10-μs flash pulse. When the length of irradiation was increased, the absorption decay kinetics changed greatly. In Figure 7, the amplitudes of the three decay components are plotted as a function of the irradiation time. The 10-ms component (M^f) had a maximal amplitude at an irradiation length of ~70 ms (Figure 7) and its amplitude decreased slightly when the irradiation length was further increased. This decrease was correlated with accumulation of N (the 1400-ms component). On the other hand, M^s with a lifetime of 350 ms developed significantly only after a large amount of N had been accumulated. When the presence of a small amount of the N-like conformer in the dark is taken into account, the accumulation kinetics of M^s is described well by the two-photon reaction: $bR_{570} \sim\!\!\rightarrow N \sim\!\!\rightarrow M^s$.

DISCUSSION

The present result shows that the protein conformation in light-adapted purple membrane is not homogeneous. First of all, a non-negligible amount of the 13-cis isomer is suggested to occur at high temperature, where the branching reaction from the trans cycle into the cis cycle may be significant. A more important phenomenon is the occurrence of O-like and N-like conformers at high temperature (Figure 1). The O-like conformer was detected only at or below neutral pH, whereas the N-like conformer occurred preferentially above pH 8. In this relation, it has been reported that there is a rapid thermal equilibrium between N and O and that this equilibrium shifts toward N at high pH (Chernavskii et al., 1889; Kouyama et al., 1988; Varo & Lanyi, 1990). Thus, the present result suggests the possibility that the back-reaction $bR_{570} \rightarrow O \rightarrow N$ is not neglected at high temperature.

To quantitatively determine how much this back-reaction is allowed, one needs to solve a related problem. We have previously proposed that the biphasic decay of M at high pH is due to a thermal equilibrium between bR_{570} and N (Kouyama et al., 1988), but our proposal of the photoreaction of N leading to a long-lived M-type product (^NM) has recently been questioned by several workers (Varo & Lanyi, 1990; Tokaji & Dancshazy, 1991). Below we discuss origins of M^s. Such discussion will be some help in understanding the late part of the trans photocycle.

(1) *Photoreaction $N \sim\!\!\rightarrow {}^NM \rightarrow bR_{570}$.* There is enough evidence that N is photoactive. First, the photoreaction of N leading to bR_{570} is supported by the observation that the conversion rate from N to bR_{570} is accelerated by background light. Second, the K-like product of N has recently been identified by Balashov et al. (1990). By using the solid-state NMR technique, Smith et al. (1989) suggested that the chromophore in ^NM has a 13-cis, N=C-syn configuration. But their assignment is recently under discussion (McDermott et al., 1991).

Varo and Lanyi (1990) disputed the existence of ^NM. Although the photoreaction leading to ^NM was originally introduced on the basis of the observation of a significant enhancement of M^s by background light, they insisted that this enhancement was due to a kinetic perturbatioin on the trans photocycle. According to their numerical calculation, the contribution of the N → M back-reaction to the M decay kinetics becomes larger with increasing intensity of the background light. To check whether their insistence is reasonable, we solved their differential equations (equations A10, 11 and 14 in their model B) by using the Runge–Kutta–Gill method at the fifth order of precision. Our calculation indicates that the apparent decay rate of M^s increases with increasing intensity of background light, but its relative amplitude scarcely changes or slightly decreases. The answer we obtained is completely different from theirs, and the discrepancy may be due to inadequate approximations used in their calculation. In any case, their explanation is inconsistent with the present observation that the background-light effect was significant even when the amplitude of M^s was negligible (<3%) in the absence of background light.

Tokaji and Dancshazy (1991) recently investigated influence of pre-excitation on the yield of M^s by the second flash. They observed that a significant enhancement of M^s was induced

even when the second flash was applied with a very short delay (100 μs), and that the influence of the pre-excitation disappeared with the same time constant as the lifetime of N. Only the latter part of their result can be explained by the photoreaction N $\sim\!\!\rightarrow$ ^{N}M. It is obvious that there is an additional mechanism of the M^{s} production. They suggested two possibilities: cooperativity between bR molecules [as reported by Ohno et al. (1981)] or heterogeneity of the ground-state conformation induced by the exciting light [as reported by Birge et al. (1989)]. Another possibility is the photoreaction of L, which may be in thermal equilibrium with M. In this relation, it has been reported that Asp-96 is partially deprotonated in L (Braiman et al., 1988). This deprotonated fraction of L might lead to an M-like state with a deprotonated Asp-96. It is also conceivable that synchronized movements of many protons generate a transient membrane potential that affects the millisecond absorption kinetics. Thus, the observation that M^{s} is already present before the rise of N does not necessarily contradict the photoreaction N $\sim\!\!\rightarrow$ ^{N}M. It is difficult without a further study to say which mechanism(s) is(are) most likely.

The question to be answered here is, How efficiently is an M-type product (^{N}M) generated upon excitation of N? The present result indicates that there is a strong correlation between the amplitude of M^{s} and the concentration of N (Figures 5 and 7). This correlation is explained readily by the photoreaction N $\sim\!\!\rightarrow$ ^{N}M. We admit that this correlation can be explained by alternative mechanisms, for instance, by assuming that the decay of M is inhibited when a neighboring bR molecule is in the N state. In the latter case, however, it is difficult to give a reasonable explanation for the observation that the amplitude of M^{f} did not decrease very much when a large fraction of bR molecules was trapped in the N state (Figure 7). On the other hand, this result is predicted by the two-photon reaction scheme (bR_{570} $\sim\!\!\rightarrow$ M^{f} $\rightarrow$ N $\sim\!\!\rightarrow$ ^{N}M $\rightarrow$ bR_{570}) in which the quantum efficiencies of the two photoreactions involved are comparable to each other.

(2) *Origins of the M^{s} Generated by Weak Excitation Light.* It is agreed that Asp-96 is a major proton donor to the deprotonated Schiff base. In the bR mutants in which Asp-96 is replaced by a nonprotonatable amino acid, the decay of M is monophasic and its decay rate is inhibited at high pH (Otto et al., 1989; Miller & Oesterhelt, 1990). In the wild-type bR, the decay rate of M^{f} is insensitive to the medium pH, and the decay rate of M^{s} is approximately proportional to the proton concentration of the medium (Ohno et al., 1981; Otto et al., 1989). The simplest explanation of the latter pH dependence is that M^{s} is generated from a ground state in which Asp-96 is deprotonated. (When Asp-96 does not work as a proton donor to the Schiff base, reprotonation of the Schiff base may occur in a similar manner as proposed for the Asp-96 mutants.) We suggest that this ground state corresponds to the N-like conformer that occurred at pH 8–10 and high temperature (Figure 1b). This assignment is supported by the observation that the amplitude of M^{s} increased significantly with increasing temperature (Figure 4a).

Recently, Balashov et al. (1991) observed that the alkali-induced red shift of the visible absorption band correlates with the appearance of the fast component in the L $\rightarrow$ M transition. They suggested that deprotonation of a tyrosine residue affects the proton-release process in the photocycle. In this relation, Varo and Lanyi (1990) have clearly indicated that the factors regulating the M formation kinetics should be distinguished from those regulating the M decay kinetics. Accordingly, the result of Balashov et al. does not contradict the hypothesis that deprotonation of Asp-96 in the ground state affects the kinetics of the proton-uptake process. Although it was previously thought that the fast M rise and the slow M decay were due to the same intermediate (Kouyama et al., 1988), this idea should be retracted. The present result (Figure 5) suggests that the rise rate of M^{s} is, in fact, much slower than that of M^{f}. (The rising phase of M^{s} is difficult to directly detect in 3 M KCl, but it becomes detectable under some solvent conditions; e.g., in 40 mM guanidine hydrochloride at pH 9.6, M^{s} develops after M^{f} largely decays.)

To understand the relationship between the N-like conformer and the N intermediate, one needs to solve related problems. First of all, the definition of N is ambiguous. Ames and Mathies (1990) introduced an intermediate N^{+} between N and O. According to their scheme, the protein (i.e., Asp-96) picks up a proton from the cytoplasm in the N $\rightarrow$ N^{+} step, and the cis-to-trans chromophore isomerization takes place in the N^{+} $\rightarrow$ O step. Obviously these two steps must be involved in the N $\rightarrow$ bR_{570} transition. But the absorption spectrum of N^{+} is unknown, so it is difficult to estimate its contribution to the temperature-induced difference spectra. Also there is no guarantee that the proton uptake always precedes the cis-to-trans isomerization; i.e., the sequence of these two steps may be reversed when the proton uptake process is strongly inhibited at very high pH. In any case, one can postulate another ground state in which Asp-96 is deprotonated and the retinal chromophore assumes the 13-trans configuration. Such a ground state seems to occur at very high pH (pH > 10), where the amplitude of M^{s} increases sharply with increasing pH, irrespective of temperature (Figure 4b). The same ground state may be formed when the protein is interacting with guanidine hydrochloride or arginine, which is known to elongate the lifetime of M at high pH (Yoshida et al., 1977; Smith et al., 1990; Nakasako et al., 1991).

(3) *Is the M $\rightarrow$ N Transition a Reversible Process?* It is shown that M^{s} and N are not kinetically coupled with each other (Figure 6). The same result has been reported previously by several workers (Dancshazy et al., 1988; Diller & Stockburger, 1988; Kouyama et al., 1988). These authors have explained M^{s} by assuming a second conformer that is in thermal equilibrium with the major conformer bR_{570}. On the contrary, a number of workers found no difference in the decay rate for M^{s} and N and they explained M^{s} by assuming the backreaction M $\rightarrow$ N (Varo & Lanyi, 1990; Ames & Mathies, 1990; Gerwert et al., 1990). The latter explanation is not justified for the following reasons.

First, Varo and Lanyi (1990) exposed samples to strong measuring light so as to operate their optical multichannel analyzer. The true decay rate of N is obtained only when the measuring light is sufficiently weak, because N is photoactive and its decay rate is accelerated by visible light. In an optical system with a sample holder positioned between a measuring light source and a monochromator, one can easily overestimate the decay rate of N; in the worst case, one may overlook its existence, as happened previously.

Second, time-resolved FTIR spectra reported by Gerwert et al. (1990) were obtained at neutral pH and low ionic strength. Under this condition, the lifetime of N is comparable to or shorter than its rise time, and the coincidental disappearance of M and N does not necessarily mean a rapid equilibrium between these states. Braiman et al. (1991) have recently pointed out that the 1555 cm^{-1} peak previously attributed to a conformational change occurring within the M intermediate is probably due to the N intermediate. There is enough evidence that the biphasic decay of M at neutral

pH is not due to the M → N back-reaction; for instance, our recent study (Fukuda & Kouyama, 1992) shows that the rate constants of both the fast and slow decay components of M increases monotonously with increasing concentration of alcohol, whereas the decay rate of N decreases.

Third, time-resolved Raman spectra reported by Ames and Mathies (1990) were obtained under such an extreme condition that approximately a half-fraction of bR molecules were excited by a pumping beam. It has recently been reported by Tokaji and Dancshazy (1991) that the relative amplitude of M^s increases with increasing intensity of actinic flash. This dependence was confirmed in the present study. As suggested earlier by Ohno et al. (1981), the cooperative mechanism in the production of M^s may not be neglected at high excitation intensity. Ames et al. (1989) previously reported that the chromophore in both M^f and M^s had a 13-cis, C=N-anti configuration, and they argued that their M^s was unlikely to be generated from N. But their M^s exhibited a mcuh larger amplitude (40%) than observed in the present study. Presumably their M^s is unrelated to the M^s generated by weak excitation light.

Although the N → M back-reaction has been described in many recent papers (Otto et al., 1989), the experimental data so far reported are explained well by alternative schemes. [In the same paper, Otto et al. described the possibility that M^s is generated from a ground state with deprotonated Asp-96.] In most cases, the N → M backreaction has been introduced on the assumption that the ground-state conformation of bR is homogeneous in light-adapted purple membrane. But this assumption is not correct at high pH or at high temperature.

It is possible that the N → M back-reaction becomes non-negligible in the presence of a very large membrane potential. But, if the N → M back-reaction were already significant in the absence of membrane potential, it would be difficult to explain the proton pumping activity of bR. Recent FTIR studies of bR mutants (Gerwert et al., 1990; Bousche et al., 1991; Pfefferle et al., 1991) have shown that proton transfer from Asp-96 to the deprotonated Schiff base takes place during the M → N transition. On the other hand, the structural analysis of bR (Henderson et al., 1990) indicates that Asp-96 and the Schiff base are separated by a thick hydrophobic layer (~10 Å). An important point is that other parts of the proton pathway in the bR molecule are more hydrophilic. If the back-reaction N → M were significant, namely, if the hydrophobic layer between Asp-96 and the Schiff base were not a large barrier against the backward proton movement, the bR molecule would behave as a proton channel.

One may argue that rapid proton movements though the hydrophilic channel can be prevented by strong hydrogen-bonding and electrostatic interactions between the chromophore and its binding pocket residues. But too strong interactions could also prevent the proton release from the Schiff base so that bR would not work as a proton pump. Needless to say, the most important subject in the study of bR is to clarify the molecular mechanism that discriminates a proton pump from a proton channel. So far, this mechanism has been described simply by using the abstract term "an irreversible switch". In the N → M back-reaction model, Varo and Lanyi (1990) postulated that this switch works during the M state ($M_1 \rightarrow M_2$). But their hypothesis is not supported by a recent dichroism experiment that shows no change in the orientation of retinal throughout the M state (Otto & Heyn, 1991).

(4) *"Protein Quake" in the M → N Transition.* We suppose that the N → M back-reaction is inhibited rigidly by the following mechanism: the protein conformation in the M state is fluctuating until the M → N transition is initiated by such a conformation change that allows a spontaneous proton transfer from Asp-96 to the Schiff base; subsequently, dielectric relaxation takes place around the Schiff base and Asp-96, making the reverse reaction difficult. This hypothesis (a "protein quake" in the M → N transition) is endorsed by the following observations: the decay rate of M is very sensitive to the medium viscosity (Beece et al., 1981; Marque & Eisenstein, 1984; Cao et al., 1991); the decay of M is slowed down in a rigid lipid environment (Fukuda et al., 1990; Jang & El-Sayed, 1989); the M → N transition is accompanied by a large change in the intensity of scattered light from a purple membrane suspension (Drachev et al., 1990).

Our idea is further supported by a recent observation that the M → N transition is accelerated when the protein conformation is softened by breaking hydrogen-bonding interactions with alcohols (Fukuda & Kouyama, 1992).

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