Hanssum, B., Dohnt, G., & Renger, G. (1985) *Biochim. Biophys. Acta 806*, 210–222.
Hoganson, C. W., & Babcock, G. T. (1988) *Biochemistry* (submitted for publication).
Hoganson, C. W., Demetriou, Y., & Babcock, G. T. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 1, pp 479–482, Martinus Nijhoff, Dordrecht.
Okamura, M. Y., Isaakson, R. A., & Feher, G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3491–3495.
Petrouleas, V., & Diner, B. A. (1987) *Biochim. Biophys. Acta 893*, 126–137.
Renger, G., & Weiss, W. (1986) *Biochim. Biophys. Acta 850*, 184–196.
Rich, P. R., & Bendall, D. S. (1980) *Biochim. Biophys. Acta 592*, 506–518.
Rutherford, A. W., & Styring, S. (1987) in *Cytochrome Systems: Molecular Biology and Bioenergetics* (Poper, S., Ed.) Plenum, New York (in press).
Saygin, Ö., & Witt, H. T. (1987) *Biochim. Biophys. Acta 893*, 452–469.
Styring, S., & Rutherford, A. W. (1988) *Biochemistry 27*, 4915–4923.
Trebst, A. (1986) *Z. Naturforsch., C: Biosci. 41C*, 240–245.
Warden, J. T., Blankenship, R. E., & Sauer, K. (1976) *Biochim. Biophys. Acta 423*, 462–478.
Weiss, W., & Renger, G. (1986) *Biochim. Biophys. Acta 850*, 173–183.
Yerkes, C. T. (1981) Ph.D. Dissertation, Michigan State University.
Yerkes, C. T., Babcock, G. T., & Crofts, A. R. (1983) *FEBS Lett. 158*, 359–363.
Yocum, C. F., & Babcock, G. T. (1981) *FEBS Lett. 130*, 99–102.
Zimmerman, J. L., & Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 416–423.

Bacteriorhodopsin Photoreaction: Identification of a Long-Lived Intermediate N (P, R_{350}) at High pH and Its M-like Photoproduct†

Tsutomu Kouyama,‡ Akemi Nasuda-Kouyama,‡ Akira Ikegami,*,‡ Mathew K. Mathew,§,∥ and Walther Stoeckenius§
Institute of Physical and Chemical Research, Hirosawa 2-1, Wako, Saitama 351-01, Japan, and Cardiovascular Research Institute, University of California, 505 Parnassus Avenue, Box 0130, San Francisco, California 94143
Received February 25, 1988; Revised Manuscript Received April 6, 1988

ABSTRACT: An alkaline suspension of light-adapted purple membrane exposed to continuous light showed a large absorption depletion at 580 nm and a small increase around 350 nm. We attribute this absorption change to an efficient photoconversion of bR_{570} into a photoproduct N (P, R_{350}), which has a major absorption maximum between 550 and 560 nm but has lower absorbance than bR_{570}. N was barely detectable at low pH, low ionic strength, and physiological temperature. However, when the thermal relaxation of N to bR_{570} was inhibited by increasing pH, increasing ionic strength, and decreasing temperature, its relaxation time could be as long as 10 s at room temperature. N is also photoactive; when it is present in significant concentrations, e.g., accumulated by background light, the flash-induced absorption changes of purple membrane suspensions were affected. Double-excitation experiments showed an M-like photoproduct of N, ^{N}M, with an absorption maximum near 410 nm and a much longer lifetime than M_{412}. It may be in equilibrium with an L-like precursor ^{N}L. We suggest that N occurs after M_{412} in the photoreaction cycle and that its photoproduct ^{N}M decays into bR_{570}. Thus, at high pH and high light intensity, the overall photoreaction of bR may be approximated by the two-photon cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightsquigarrow (^{N}L \leftrightarrow {}^{N}M) \rightarrow bR_{570}$, whereas at neutral pH and low light intensity it can be described by the one-photon cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightarrow O_{640} \rightarrow bR_{570}$. The result of light-induced pH changes in purple membrane suspensions suggested that one proton is taken up from the medium during the thermal relaxation $N \rightarrow bR_{570}$ or the light reaction $N \rightsquigarrow bR_{570}$ (not during the reaction $M_{412} \rightarrow N$). At high pH and high ionic strength, a small amount of N appears to also be present in the dark, which implies that a thermal backreaction from bR_{570} to N also exists. The proposed modification of the photoreaction cycle model will require confirmation and possibly corrections by other techniques, e.g., vibrational spectroscopy. However, as it stands, it offers a satisfactory explanation of a variety of earlier observations which are inconsistent with the simple, generally used model $bR \rightsquigarrow K \rightarrow L \rightarrow M \rightarrow O \rightarrow bR$.

Bacteriorhodopsin (bR),[1] a transmembrane protein found in halobacteria, contains one molecule of retinal bound to the ε-amino group of a lysine residue via a protonated Schiff base linkage. Binding to the protein shifts the retinal absorption maximum >100 nm to the red and generates a broad absorption band near 570 nm. In the light, bR translocates protons across the cell membrane and generates an electric potential and a pH gradient which can be as large as 4 pH units (Stoeckenius et al., 1979; Kouyama et al., 1987). Preilluminated (light-adapted) bR contains only *all-trans-*

† Work in Japan supported by special coordination funds for promotion of science and technology, by a research grant from the Agency of Science and Technology of Japan entitled "Solar Energy-Photosynthesis", and, in part, by Grants-in-Aids from the Ministry of Education, Science, and Culture of Japan. Work in San Francisco supported by National Institutes of Health Grant GM-27057.
‡ Institute of Physical and Chemical Research.
§ University of California.
∥ Present address: Division of Biology, 216-76, California Institute of Technology, Pasadena, CA 91125.

[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; M^f, fast decay component; M^s, slow decay component.

0006-2960/88/0427-5855$01.50/0

retinal; but in the dark, an equilibrium of the 13-cis and all-trans isomers is slowly established.

The cyclic photochemical reaction of bR containing the all-trans chromophore, which drives the proton translocation, has a half-time of ~7 ms under physiological conditions. It has been extensively studied with time-resolving spectroscopic techniques in purple membrane (pm), membrane fragments consisting of two-dimensional crystals of bR and lipid. A transient isomerization of the retinal C13–C14 double bond and deprotonation/reprotonation of the Schiff base take place during this photoreaction cycle (Lewis et al., 1974; Pettei et al., 1977; Tsuda et al., 1980; Smith et al., 1985), but details of the photocycle and the coupling to the proton pumping are still poorly understood. In the original scheme (Lozier et al., 1975; Lozier & Niederberger, 1977), the trans photocycle contained five spectroscopically distinct photointermediates, K_{590}, L_{550}, M_{412}, N_{530}, and O_{640} (numbers indicates the estimated absorption maxima) with lifetimes in the nanosecond to millisecond range. The existence of N_{530} was never rigorously established; but, two forms of M and/or L, distinguishable by their decay kinetics, and/or dichroism but not by their absorption maxima, have been repeatedly reported (Slifkin & Caplan, 1975; Eisenbach et al., 1976; Hess & Kuschmitz, 1977; Ohno et al., 1981; Groma et al., 1986). Recently, additional kinetic components and/or photointermediates, e.g., P and R_{350}, which are characterized by long lifetimes, have been described (Xie et al., 1987; Drachev et al., 1986; Dancshazy et al., 1986). The origin of multiple kinetic components in the decay of M and L with different sensitivity to environmental conditions has remained controversial (Parodi et al., 1984). For a general description of the photocycle kinetics, see Nagle et al. (1982).

We have studied the photoreactions in the millisecond and subsecond time range under conditions which emphasize these controversial absorption changes. We shall show here that the absorbance changes attributed to N, P, and R_{350} can all be explained by the existence of one photocycle intermediate located between M_{412} and O_{640} in the bR photocycle. It has an absorption maximum between 550 and 560 nm but a lower extinction than bR_{570}. Its decay at high pH and low temperature determines the slow recovery of bR_{570} absorbance; its photoproducts resemble L_{550} and M_{412} and contribute the slowest M decay component.

Materials and Methods

Purple membrane fragments from *Halobacterium halobium* JW-3 were prepared according to the established procedure (Oesterhelt & Stoeckenius, 1974).

Absorption spectra in the presence of intense actinic light were measured with a cross-illumination spectrophotometer. The principle of measurement has been described previously (Kouyama et al., 1985). Briefly, monitoring beam and actinic beam were alternately separated with a mechanical chopper system (Figure 1). Three chopper plates, each with two holes and two reflecting surfaces, were aligned and rotated synchronously at 5000–12000 rpm. An Xe lamp (300 W) with a parabolic mirror was used as light source for the actinic beam (L_a in Figure 1), which was passed through a heat-absorbing water filter, reflected from a cold mirror (400–700 nm), passed through optical filters, and then focused into the sample holder. The time-averaged intensity of the actinic light at the sample holder was measured with an actinometer (Advantest TQ8210). A spectrophotometer with double monochromator (Shimadzu UV350a, Kyoto) provided the monitoring beam. The current signal from a photomultiplier tube (Hamamatsu R374, Hamamatsu Japan) was sent to a logarithmic amplifier

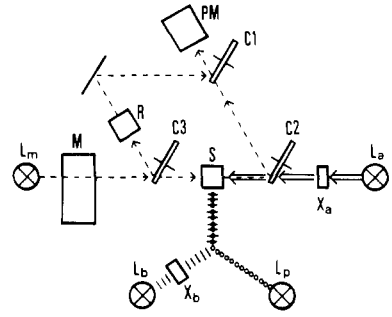

FIGURE 1: Design of the spectrophotometer. Light sources for actinic beam (L_a), measuring beam (L_m), background light (L_b), and flash lamp (L_p). (C_1–C_3) Chopper plates; in the actual instrument, C_2 consists of two chopper plates, and all four chopper plates are mounted coaxially to assure synchronous rotation at high speed (15000 rpm). (M) Double monochromator. (PM) Photomultiplier tube. (X_a and X_b) Mechanical shutters. (S) Sample cell holder. (R) Reference cell holder.

and then to two sample/hold amplifiers which were operated under the supervision of gating signals from the choppers. The difference between the output signals from the sample/hold amplifiers, which is proportional to the difference in absorbance of the sample and reference cell (S and R in Figure 1), was digitized and stored in the memory of a personal computer (NEC PC9801/VM2, Tokyo). Subsecond absorption kinetics were measured with a mechanical shutter (X_a in Figure 1) which was set in the optical path of the actinic light. Time resolution was a few milliseconds at the maximal chopper frequency of 400 Hz.

Measurements of millisecond absorption kinetics were carried out by attaching a constant-power Xe flash lamp (Nissin Electronic Co., Ltd., Tokyo; L_p in Figure 1) to the spectrophotometer. In this measuring mode, the chopper positions were fixed, and appropriate interference filters protected the photomultiplier tube. The light pulses of 10-μs width were passed through optical filters (>540 nm) and irradiated the sample cell uniformly at right angle to the monitoring beam. Repetition rate of the light pulses was usually 0.25 Hz and, if necessary, was reduced to 0.1 Hz. The light intensity of each pulse was reduced to the level at which only a few percent of the pigment was excited. The amplified photocurrent was fed to a digital memory scope (Iwatsu DS6121, Tokyo) operated in a pretrigger mode. For flash-photolysis experiments in the presence of background light, emission from a 300-W projector lamp (L_b) was passed through an infrared cutoff filter (>660 nm) and a mechanical shutter (X_b) and focused onto a small mirror which was set into the optical path of the pulsed light so that the constant light irradiated the sample cell uniformly.

Results

Subsecond Absorption Kinetics of pm Suspensions. At alkaline pH (>8), the light-induced changes in the absorption spectrum of light-adapted pm strongly depended on the actinic light intensity. Figure 2a shows spectra of pm exposed to different intensities of orange light (540–700 nm). Weak actinic light (0.5 mW/cm^2) caused a depletion in the visible absorption band but only a negligible absorption increase near 400 nm. At such a low light intensity, the absorption recovery at 580 nm after 1-s illumination was approximated by a single-exponential function with a time constant in the 0.1–10-s range (trace 2 in Figure 2b). At high actinic light intensity (>2 mW/cm^2), a significant absorption increase near 410 nm accompanied the 570-nm decrease, and in parallel with the development of this band, a faster component in the 10–

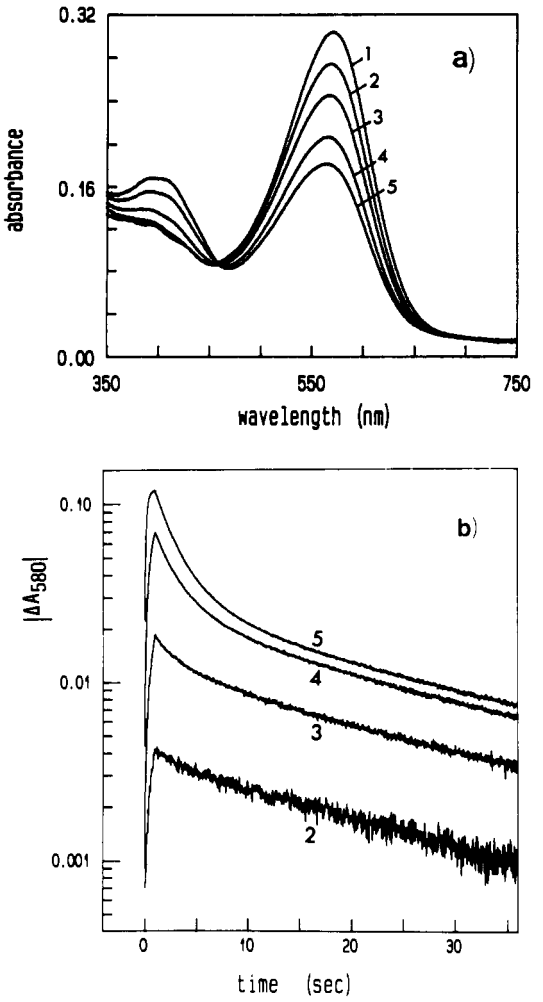

FIGURE 2: (a) Absorption spectra of an alkaline suspension of light-adapted pm without (trace 1) and with increasing amounts (traces 2–5) of orange light (540–700 nm). The spectra were measured at a chopper frequency of 200 Hz with water in the reference cell. (b) Absorption changes at 580 nm induced by 1-s illumination with orange light beginning at time 0. As the base line for calculating time constants, we used the absorbance observed before the illumination. The time-averaged intensity of actinic light in (a) and (b) was 32 (traces 5), 8 (traces 4), 2 (traces 3), or 0.5 mW/cm^2 (traces 2). Sample: 3 M KCl and 10 mM bicarbonate, pH 10.35, at 10 °C.

1000-ms range appeared in the 570-nm recovery (Figure 2b). Thus, both time constants are significantly slower than any seen in the photocycle at low ionic strength and neutral pH.

In Figure 3a, the amplitude of the slow recovery component is plotted against the wavelength of measuring light (open squares), and the dotted line is the light-induced difference spectrum obtained with very weak actinic light. Both spectra are indistinguishable and characterized by a large but relatively narrow negative peak at 580 nm and small positive peaks in the near-UV region. In the visible region, the difference spectra are very similar to the difference spectrum associated with a slow intermediate P recently reported by Drachev et al. (1986). In the near-UV region, the difference spectra have the largest positive peak at 350 nm, characteristic for the slow intermediate R_{350} recently described by Dancshazy et al. (1986). We assume that both absorbances are due to the same intermediate whose main absorption band in the visible is slightly weaker and blue shifted with respect to that of bR_{570} (see Discussion). We shall refer to it provisionally as N (P, R_{350}) for reasons that will become obvious later (see Discussion).

At neutral pH and at low ionic strength, the slow recovery component was not observed due to its accelerated decay.

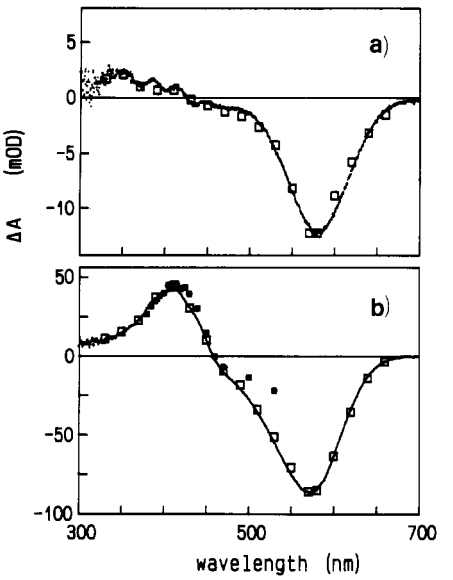

FIGURE 3: Amplitudes of the slow (a) and fast (b) decay components in the subsecond absorption kinetics. The data shown by the open squares were obtained from analyses of the absorption recoveries observed after 1-s illumination with orange light (32 mW/cm^2, 540–700 nm). The dotted line in the upper panel was obtained by subtracting the absorption spectrum observed in the presence of weak orange light (0.5 mW/cm^2, 540–700 nm) from the absorption spectrum observed ~1 min after the actinic light was turned off. The dotted line in the lower panel is the difference for high (32 mW/cm^2) and intermediate (8 mW/c m^2) actinic light intensity where the contribution of the slow decay component is negligible (<5%) because under the solvent conditions used (3 M KCl, at pH 9.7, at 25 °C) its amplitude was already very close to its maximum at 8 mW/cm^2. The closed squares in the lower panel show the inverted amplitude of a very fast decay component (1 ms) seen in the millisecond absorption kinetics observed after a 10-μs flash (>580 nm).

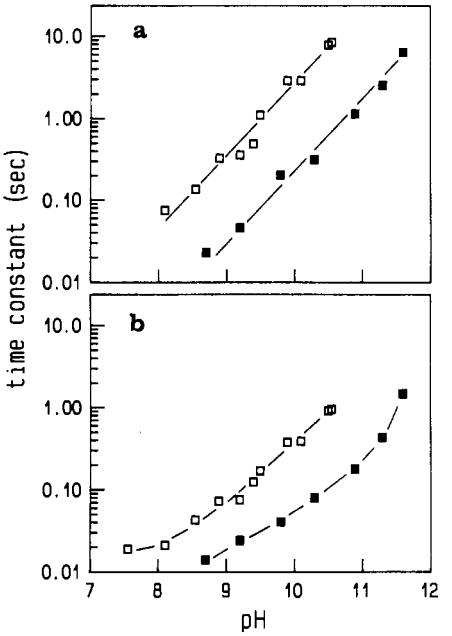

FIGURE 4: (a) pH dependence of N (P, R_{350}) decay. The time constants were determined from the absorption recovery at 580 nm after a weak actinic light was turned off, which caused no significant absorption change at 410 nm. (b) pH dependence of the ^{N}M decay. The time constants were obtained from the absorption recovery at 410 nm after strong actinic light (32 mW/cm^2) was turned off. The open squares and closed squares represent the data obtained at high ionic strength (3 M KCl) and at low ionic strength (10 mM bicarbonate), respectively. Temperature: 25 °C.

Figure 4a shows the pH and ionic strength dependences of the slow time constant. With increasing pH, the time constant

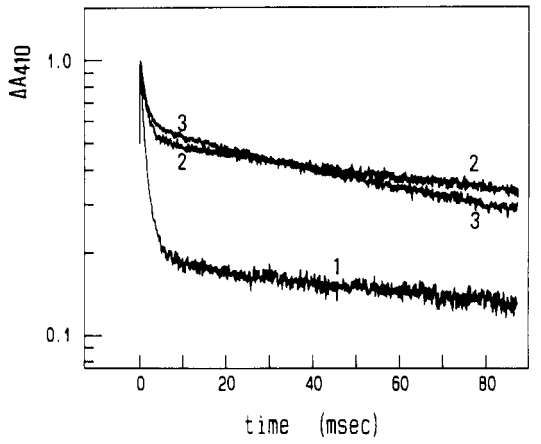

FIGURE 5: Flash-induced absorption changes at 410 nm in the absence (trace 1) and in the presence (traces 2 and 3) of orange light (540–660 nm). To calculate time constants, we used as a base line the absorbance observed just before the light flash. The light intensity of background light was 1 (trace 2) or 4 mW/cm^2 (trace 3). Sample: 10 μM bR, 3 M KCl, and 10 mM bicarbonate, pH 9.55, at 25 °C.

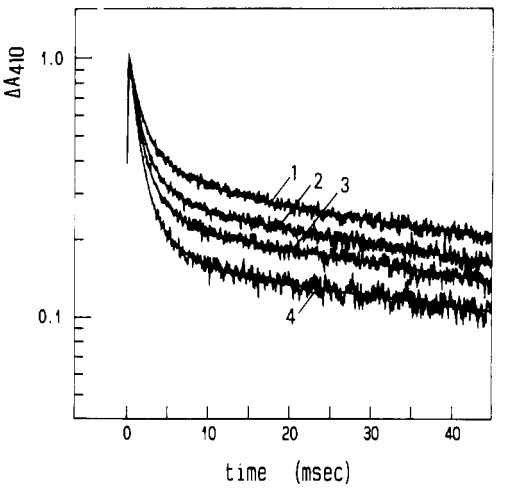

FIGURE 6: Flash-induced absorption changes at 410 nm after preillumination. The pm suspensions were exposed to orange light (1 mW/cm^2, 540–660 nm) for 2.5 s; then, flashes (540–660 nm) were triggered with 50-, 200-, or 400-ms delay (traces 1–3). Trace 4 was obtained without preillumination. Sample: 10 μM bR, 3 M KCl, and 10 mM bicarbonate, pH 9.3, at 25 °C.

increased exponentially; i.e., the decay rate increases linearly with the concentration of free H^+. The presence of 3 M KCl decreased the decay rate by a factor of ~10. At neutral pH and at low ionic strength, the lifetime of N (P, R_{350}) became comparable to, or shorter than, the lifetime reported for M_{412} or O_{640} intermediates. The decay rate also showed a strong temperature dependence; a 10 °C increase in temperature resulted in an increase by a factor of 3 (not shown).

The wavelength dependence of the amplitude of the fast recovery component is shown by the open squares in Figure 3b. Essentially the same spectrum, but with more fine structure, was obtained as the photo-steady-state difference spectrum at high actinic light intensity (dotted line in Figure 3b). The broad negative peak at 570 nm and a positive peak at 410 nm with a cross over near 460 nm are characteristic for the photoconversion of bR_{570} into M_{412}. However, a significant amount of this "M-like" intermediate could be accumulated only when pm was irradiated with strong actinic light. We shall refer to this intermediate as ^{N}M for the following reason. The amplitude of the fast recovery component at lower light intensities was almost proportional to the square of the intensity, whereas it showed nearly linear dependence at high intensities, when the amplitude of the slow recovery component approached a maximum. This result suggested that the accumulation of ^{N}M was generated by excitation of N (P, R_{350}) and that it decayed to bR_{570}.

Millisecond Absorption Kinetics of pm Suspensions. As has been reported previously (Ohno et al., 1980), at high pH and ionic strength, absorption decay kinetics at 410 nm in the millisecond time range are approximated by two time constants: a time constant of ~1 ms and a much longer one. We measured the millisecond absorption kinetics and found that background illumination increased the extent of the slow decay component (M^s) (Figure 5). In the absence of background light, the fast decay component (M^f) was predominant. When pm was excited with weak light pulses at a low repetition rate (0.25 Hz or lower) and without background illumination, that is, when a long-lived N (P, R_{350}) did not accumulate, more than 80% decayed via M^f at any solvent condition investigated. In the presence of background light (540–660 nm), the slow decay component (M^s) could account for as much as 60% of the decay. The extent of this enhancement depended on the solvent condition as well as on the intensity of background light. At a given solvent condition (i.e., pH 9.5 in 3 M KCl at 25 °C), the enhancement leveled off at the intensity (4 mW/cm^2) at which the photo-steady-state amount of N (P, R_{350}) due to the background light reaches a maximum. (The small increase in the M^s decay rate at the highest intensity will be discussed later.) At a given intensity of background light, the extent of M^s increased with increasing pH and correlated strongly with the accumulation of N (P, R_{350}).

The correlation between N (P, R_{350}) and M^s was confirmed by the double-excitation experiments shown in Figure 6. An actinic light pulse was applied after the background light was turned off. With increased delay time, the amplitude of M^s decreased and finally reached the level observed in the absence of background light; the decrease rate matched the rate of slow absorbance recovery at 580 nm in the subsecond kinetics. At the solvent condition shown in Figure 6, the relaxation time was 0.3 s, which was also the time constant for the decay of N (P, R_{350}). We assume that the nonzero amplitude of the slow component in the 410-nm phototransients in the absence of background light is due to a thermal equilibrium between N (P, R_{350}) and bR_{570} (see Discussion).

The decay time constant of the slow component (M^s) seen in the millisecond absorption kinetics was approximately the same as that of the fast recovery component due to ^{N}M seen in the subsecond absorption kinetics (the open squares in Figure 4b versus the open squares in Figure 7). Thus, M^s is apparently due to the decay of ^{N}M, and we ascribe M^f to the decay of M_{412}.

The decay time constant of M^s showed a strong pH dependence, whereas little pH dependence was seen for M^f (Figures 4b and 7). Strong background light increased the rate of M^s (traces 2 and 3 in Figure 5). Since the acceleration of M^s decay was induced by orange background light (540–660 nm) which would not be effectively absorbed by ^{N}M, it seems very likely that ^{N}M dynamically equilibrates with another photoproduct, ^{N}L, which absorbs the long-wavelength light, and may be similar in its absorption spectrum to L_{550} (see Discussion).

Light-Induced pH Changes in Purple Membrane Suspensions. The pH changes in pm suspensions show that, during the decays of both N (P, R_{350}) and ^{N}M, protons are taken up. In Figure 8, time-resolved transmission changes at 580 nm and light-induced pH changes of a pm suspension are recorded. After strong orange light was turned off, the pH and transmission changes showed two time constants: the slower cor-

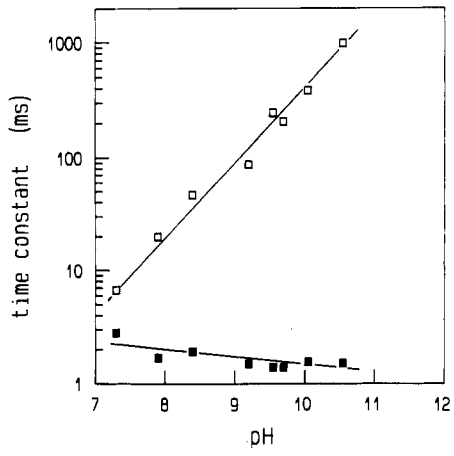

FIGURE 7: pH dependence of the M_{412} (M^f) and ^NM (M^s) decays. The time constants were obtained from the 410-nm absorption changes 50 ms (500 ms for the data above pH 10) after a 10-μs light flash (540–660 nm). An optimal fit to the data required three exponential functions, but the faster two differed from each other only by a factor of 2 or 3 (0.6–2 ms), whereas the time constant of the slowest one was 10–1000 times longer; the closed squares represent the weighted average of the two faster time constants. Sample: 10 μM bR, 3 M KCl, and 10 mM bicarbonate, at 25 °C.

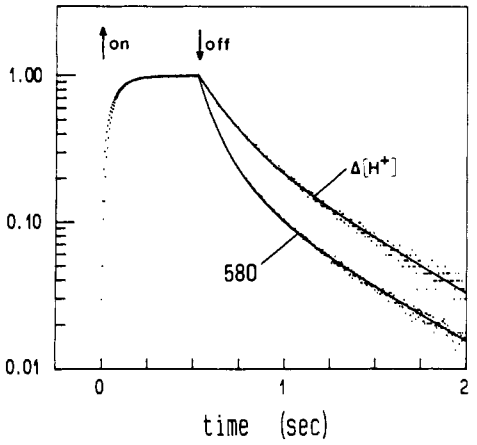

FIGURE 8: Light-induced pH and absorption changes in pm suspension. The sample contained 0.2 mM brilliant yellow. It was exposed to orange actinic light (32 mW/cm^2) in a cell with 1-mm optical path length at a chopper frequency of 400 Hz. The pH change in the medium was estimated from the absorption change at 457 nm, where the contribution from the photoreaction of bR is negligible; illumination caused acidification of the medium. The transmission changes at 580 nm reflect the bleaching and recovery of bR_{570}. The rapid pH decrease caused by the actinic light relaxes with two time constants, 594 (±30) and 162 (±20) ms, and the extent of the slower component increased relative to that of the faster, at lower actinic light intensities. Similar time constants (583 ± 40 and 151 ± 30 ms) with the same intensity dependence were found in the absorption recovery kinetics at 580 nm. An additional component with a time constant of 65 ± 20 ms in the absorption kinetics decreased even faster with the actinic light intensity than the 151-ms component. Sample: 50 μM bR and 0.2 M KCl, pH 8.5, at 10 °C.

responded to the thermal decay time constant of N (P, R_{350}) (594 ± 30 ms) and the faster to the decay time constant of ^NM (162 ± 20 ms). The 580-nm transmission change showed a third, still faster time constant (65 ± 20 ms), which, at lower light intensities, rapidly decreases in extent relative to the slower processes. We tentatively attribute it to another unidentified photoproduct from an intermediate, possibly ^NL.

Discussion

As a tentative interpretation of the presented results, we construct the model for photoreaction of bR shown in Figure 9: (1) In light-adapted pm at high pH and ionic strength,

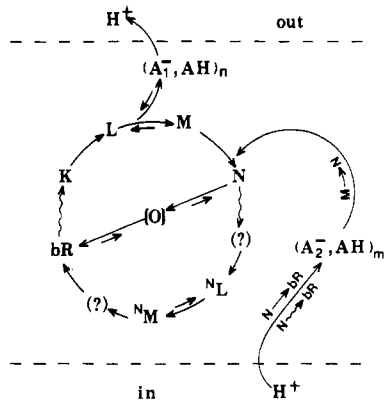

FIGURE 9: A tentative model for the photoreaction cycle and proton translocation of bR. Light reactions are indicated by wavy arrows. At low ionic strength and neutral to moderately acidic pH, the decay of N is comparable to or faster than its rise time, and O becomes the dominant intermediate. At high pH and high ionic strength, the decay of N becomes so slow that O decays faster than it rises and is no longer observed. Under the same conditions, the thermal backreaction bR → N becomes significant. The proton exchanges with A_1^- and A_2^-, which are connected to opposite sides of the membrane, probably involve more than one protein residue.

bR_{570} is the predominant ground state of bR, but there exists also a small amount (5–20%) of a second conformer N (P, R_{350}), which has a slightly blue-shifted absorption maximum and slightly less extinction. (2) Excitation of bR_{570} generates M_{412} via intermediates K and L; M_{412} then decays into N (P, R_{350}) within a few milliseconds (M^f). (3) The thermal relaxation of N (P, R_{350}) via O_{640} into bR_{570} is relatively fast at neutral or acid pH but takes seconds at high pH. (4) Excitation of N (P, R_{350}) generates an intermediate ^NM via ^NL; ^NM has an absorption spectrum indisitinguishable at the present resolution from M_{412} but it decays more slowly (M^s) to bR_{570}. (5) Similar to L and M, ^NL and ^NM are in thermal equilibrium. (6) Before the formation of N (P, R_{350}), protons are released into the medium in the L → M transition; during the decay of N (P, R_{350}) as well as during the decay of ^NM, protons are taken up. The model, while still speculative, has the advantage over many other proposed modifications of the "classic" photoreaction scheme in that it explains not only the new data presented here but also many earlier observations for which different modifications of the photocycle model have been proposed. We shall discuss some of these as we xamine arguments for the different intermediate reactions proposed. For this discussion, it should be kept in mind that increasing the ionic strength is equivalent to increasing the pH, because increasing the salt concentration increases the surface pH of the membrane, which, because of the high negative charge density, is substantially below the pH of the medium.

The Light-Driven Transitions bR_{570} ⇝ M_{412} and N (P, R_{350}) ⇝ ^NM. Two kinetic components of M decay, which have usually been called the fast (M^f, in the 1–10-ms range) and the slow (M^s, in the tenths to hundredths of a millisecond range), have often been observed, and Mathew et al. (1985) have reported slightly different absorption maxima for the two components. The explanations which have been advanced mostly assumed a branching reaction at an earlier step in the photocycle. The novel feature of our interpretation is that they are due to the decay of two different intermediates, M_{412} and ^NM, generated by excitation of two different forms of bR, bR_{570} and N (P, R_{350}), respectively; the latter can accumulate as a slowly decaying photocycle intermediate, but at high pH, a small amount is also generated thermally. Its visible absorption spectrum is similar to that of bR_{570}, but it has a slightly higher absorbance around 350 nm. The main evidence

for this interpretation is the linear dependence, on the amount of N (P, R_{350}) accumulated under background illumination, of the amplitude of the slow decay component, M^s, in the 410-nm absorption transient during the decay of a preestablished photo-steady-state equilibrium between N (P, R_{350}) and bR_{570}.

The dependence of the decay kinetics of M on the intensity or the duration of light pulses was previously observed by several other groups (Korenstein et al., 1979; Ohno et al., 1981). Ohno et al. observed that the amplitude of M^s was proportional to the square of the intensity of the light pulse. At that time the existence of a long-lived N (P, R_{350}) intermediate absorbing in the same region as bR_{570} was not considered, and the authors explained their observations by assuming cooperativity among neighboring bR molecules. A more recent analysis, however, eliminated cooperativity as a possible explanation (Xie et al., 1987).

The present proposal is also consistent with the previous observation that the picosecond absorption kinetics of bR were greatly altered when a flow system of pm was used so that a long-lived intermediate did not accumulate (Gillbro & Sundstrom, 1983). Gillbro and collaborators were the first to postulate the existence of a long-lived photoproduct with an absorption spectrum similar to that of bR (Gillbro et al., 1977; Gillbro & Kriebel, 1977). They named it pseudo-bR (P-bR). It is, however, not obvious how their low-temperature data can be correlated with our present observations.

The Transition $M_{412} \rightarrow N$ (P, R_{350}). This transition is required to explain the efficiency of N (P, R_{350}) formation. On the assumptions that the amount of N (P, R_{350}) existing in light-adapted bR is negligibly small, that it is the only photoproduct accumulating in weak actinic light, and that the sample is optically thin, the initial rate of increase in the concentration of N (P, R_{350}) after the 540 (±6) nm actinic light is turned on, $(d[N]/dt)/[bR]$ at $t = 0$, is given by $\phi_N\delta_{bR}(540)I(540)$, where ϕ_N is the quantum yield of N (P, R_{350}) formation, $\delta_{bR}(540)$ is the cross section of bR_{570} at 540 nm, and $I(540)$ is the actinic light intensity. The initial rate of absorption change at 580 nm, $-(dA/dt)/A$ at $t = 0$, will be $\phi_N\delta_{bR}(540)I(540)\ [\epsilon_{bR}(580) - \epsilon_N(580)]/\epsilon_{bR}(580)$, where ϵ_{bR} and ϵ_N are the molar absorption coefficients of bR_{570} and N (P, R_{350}), respectively. At the actinic light intensity of 0.46 mW/cm^2, the initial rate of absorption change at 580 nm was 0.037 s^{-1}, and from this rate we obtained

$$\phi_N[\epsilon_{bR}(580) - \epsilon_N(580)]/\epsilon_{bR}(580) = 0.13 \qquad (1)$$

We corrected for the inner-filter effect ($OD_{540} = 0.2$) and the contribution (~30%) of the N (P, R_{350}) $\rightsquigarrow$ ^NM transition to the absorption change; but the existence of a small amount of N (P, R_{350}) in light-adapted bR was not taken into account, and correction for it would result in a slightly larger value for $\phi_N(\epsilon_{bR} - \epsilon_N)/\epsilon_{bR}$. To determine the lower limit of ϕ_N, we obtained an absorption spectrum of N (P, R_{350}) by adding bR_{570} absorbance to the difference spectrum of Figure 3a until the resulting spectrum was positive at all wavelengths (Figure 10). This gave a minimal value of 0.55–0.6 for the ratio ϵ_N/ϵ_{bR} at 580 nm. Substituting this into eq 1 and solving for ϕ_N gives a minimal value of >0.3. For the quantum efficiency of M_{412} formation, ϕ_M, we obtained a value of 0.6 for pm suspensions in ether (+3 M KCl) in which the decay of M_{412} was monophasic, consistent with the corrected value recently reported by Oesterhelt's group (Oesterhelt et al., 1985). Bogomolni et al. have reported earlier that ϕ_M for pm suspensions in water and high salt/ether is the same (Bogomolni et al., 1980). A recent redetermination of ϕ_K also yields a value >0.5 (A. Xie and R. A. Bogomolni, personal communication),

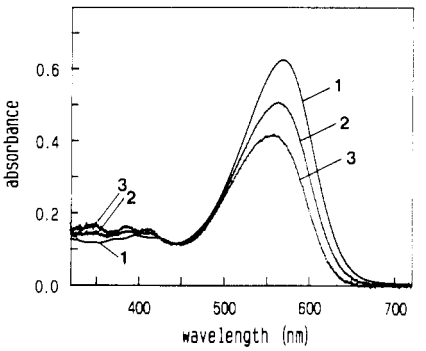

FIGURE 10: Absorption spectrum of N is calculated from the difference spectrum from Figure 3a (dotted line) and the absorption spectrum of light-adapted pm (trace 1). Trace 2 shows the spectrum obtained when ϵ_N/ϵ_{bR} at 580 nm was assumed to be 0.78 as expected for the quantum yield $\phi_N = 0.6$. The trace 3 shows the spectrum obtained when ϵ_N/ϵ_{bR} at 580 nm had the minimal value of 0.6. In these calculations, a small amount of N existing in light-adapted pm was neglected.

and the quantum efficiency for H^+ translocation requires a value of 0.6 for quantum efficiency of the photoreaction cycle if one proton is translocated per cycle (Bogomolni et al., 1980), as recent data indicate (Drachev et al., 1986; Grzesiek & Dencher, 1986). Therefore, ϕ_N is comparable to ϕ_M, and their sum is close to 1.0 or larger, and therefore, N (P, R_{350}) is likely to be or must be an intermediate occurring after M_{412}.

Flash-induced absorption changes in the long-wavelength region also suggested the transition $M_{412} \rightarrow$ N (P, R_{350}). At 530 nm, the slow absorption recovery component associated with the thermal decay of N (P, R_{350}) had an amplitude about half as large as that of the 1-ms recovery component (M^f). Apparently, the same slow component with a significant amplitude was also seen by others [e.g., Ort and Parson (1978), Drachev et al. (1986), and Dancshazy et al. (1986)]. Drachev et al. also attributed it to the decay of their P intermediate, which they too placed in the photoreaction cycle between M_{412} and bR_{570}. Dancshazy et al. observed the very slow recovery component at 570 nm and found a corresponding decay of absorbance at 350 nm. They called their intermediate R_{350} and proposed that R_{350} and the two forms of M are formed in parallel. We have shown here that R_{350} is apparently identical with N (P, R_{350}). Therefore, it has a large absorption band in the visible region, and their model would require $\phi_N + \phi_M > 1$. The model in which N (P, R_{350}) is a product of M_{412} avoids this implausible conclusion. In this case, the amplitudes of the 1-ms component (M^f) are proportional to $\epsilon_N - \epsilon_{ML}$ (not $\epsilon_{bR} - \epsilon_{ML}$), where ϵ_{ML} is a weighted average of the molar extinction coefficients of M_{412} and L_{550}, which are supposed to be in dynamic equilibrium. According to the results reported by Alshuth and Stockburger (1986), the rate constant of the backreaction M_{412} to L_{550} is about one-third of that of the forward reaction; then, the ratio $\epsilon_{ML}/\epsilon_{bR}$ at 530 nm takes a value of 0.2–0.3. Combined with the ratio ϵ_N/ϵ_{bR} of 0.7–0.85, their result suggests that the difference $\epsilon_N - \epsilon_{ML}$ is relatively small, only 40–65% of ϵ_{bR}. Thus, at the long wavelength, the difference $\epsilon_N - \epsilon_{ML}$ (i.e., the amplitude of the 1-ms component) is no longer very large as compared to the difference $\epsilon_{bR} - \epsilon_N$ (i.e., the amplitude of the very slow component). Thus the relation $\phi_N \sim \phi_M$ can also be derived from the observed absorption kinetics. In addition, the difference spectrum associated with the 1-ms component (the closed squares in Figure 3b) is characterized by a relatively large positive band around 410 nm compared to the negative band in the visible region. This profile is expected from the difference spectrum $\epsilon_N - \epsilon_{ML}$ but not from the difference spectrum

$\epsilon_{bR} - \epsilon_{ML}$, again suggesting that M_{412} decays to N (P, R_{350}).

Other similar but independent arguments can be derived from our own observations and data published by others but will not be presented here. Below we will show that the chromophore conformational changes detected by retinal extraction and more conclusively by vibrational spectroscopy also require a N (P, R_{350}) intermediate after M_{412} (see Isomerizations of the Retinal Chromophore).

The Decay $^{N}M \rightarrow bR_{570}$. This pathway explains the millisecond absorption kinetics at 410 and 570 nm in the presence of strong background light. The extent of the 1-ms decay component (M^f) remained significant, even when the intensity of the background light was so high that the photo-steady-state concentration of N (P, R_{350}) had almost reached a maximum; at these high light intensities, the amplitude of M^f and M^s became approximately equal (Figure 5). Long-wavelength excitation induces the slow decay component M^s only when N (P, R_{350}) is present, and the 410-nm decay is then matched by a 570-nm recovery component, strongly suggesting the pathway N (P, R_{350}) $\rightsquigarrow$ $^{N}M \rightarrow bR_{570}$.

The shape of the difference spectrum shown by the open squares in Figure 3b, the cross-over point at 460 nm, and the position of the depletion maximum also suggest that ^{N}M decays to bR_{570}. This profile fits the spectrum $\epsilon_{^{N}ML} - \epsilon_{bR}$ better than $\epsilon_{^{N}ML} - \epsilon_{N}$, where $\epsilon_{^{N}ML}$ represent the absorption spectrum of a dynamically equilibrated state between ^{N}M and ^{N}L.

The Process N (P, R_{350}) $\rightarrow$ *(O_{640})* $\rightarrow$ *bR_{570}*. If the O_{640} intermediate is positioned between N (P, R_{350}) and bR_{570} and if N (P, R_{350}) is replaced by N_{530}, the upper part of the scheme in Figure 9 is identical with the scheme proposed by Lozier et al. in 1975 (Lozier et al., 1975; Lozier & Niederberger, 1977). A strong correlation exists between the lifetime of N (P, R_{350}) and the appearance of O_{640}. O_{640} increases at a lower pH, at a lower ionic strength, and at a higher temperature, whereas the lifetime of N (P, R_{350}) or N increases with increasing pH, with increasing ionic strength, and with decreasing temperature. Experimentally, O_{640} is detectable only when the lifetime of N or N (P, R_{350}) is short, because if, in the thermal relaxation N $\rightarrow$ O $\rightarrow$ bR_{570} at or below neutral pH, the lifetime of N becomes shorter than that of M_{412}, N disappears; this explains the observation that the rise rate of O_{640} is almost identical with the decay rate of M_{412} (Lozier et al., 1975; Lozier & Niederberger, 1977). At high pH, the lifetime of N becomes so long that any short-lived intermediate occurring after N is no longer detectable.

N (P, R_{350}) is spectroscopically indistinguishable from N_{530} and fits into the same position in the photocycle. Using a value between 0.6 and 0.78 for the ratio ϵ_N/ϵ_{bR} at 580 nm, we calculated an absorption maximum between 550 and 560 nm. The absorption spectrum of N_{530} was originally obtained from spectra containing large contributions from M, L, and probably also ^{N}M and ^{N}L. The calculated maximum is also sensitive to the decay parameters used in its analysis. We therefore consider the apparent discrepancy in the absorption maxima for N (P, R_{350}) and N_{530} insignificant and conclude that we are dealing with the same intermediate, and we shall from now on refer to it simply as N, because this was the designation under which it was first described and which logically follows from its position in the sequence of intermediates.

The Dynamic Equilibrium $^{N}M \leftrightarrow {}^{N}L$. This reaction was introduced to explain the observation that the decay component M^s is accelerated by strong orange background light (Figure 5). This observation suggests that ^{N}M is in thermal equilibrium with a photoreversible precursor which has an absorption maximum above 500 nm and, in analogy to the bR photoproduct, is designated ^{N}L. Since no positive peak is seen at the red edge of the difference spectrum $\epsilon_{^{N}ML} - \epsilon_{bR}$ (the open quares in Figure 3b), the absorption maximum of ^{N}L should be blue shifted as compared to that of bR_{570}. The small shoulder above 500 nm in the depletion band (Figure 3b) is consistent with this interpretation. Raman spectra at pH 10.5 also indicated a dynamic equilibrium between a slowly decaying M and a long-lived "L-like" precursor (Alshuth & Stockburger, 1986), which seems to be identical with our ^{N}L or N.

The Dark Reaction $bR_{570} \rightarrow N$. Figure 9 shows a thermal reaction $bR_{570} \rightarrow$ N; it explains the small but finite amplitude of the slow decay component in the 410-nm phototransients observed in the absence of background light, which did not disappear when the intensities of the measuring and actinic light were reduced. We found that the relative amplitude of the slow component tended to become smaller when longer wavelength actinic light was used. Since N absorbs maximally at shorter wavelengths than bR_{570}, this is expected if a significant amount of N is present in light-adapted pm at high pH. Its presence at high pH in the dark is also supported by earlier observations of a slow component in the M decay which appears above pH 9.0 under conditions where nanosecond actinic light pulses are applied at low frequency, so that excitation of N generated by the exciting pulse is precluded (Scherrer & Stoeckenius, 1985). In addition, in the same pH range, a fast component (6 μs) appears in the M-rise kinetics, presumably due to the rise of ^{N}M [the fast M rise and the slow M decay have been suggested to be due to the same intermediate (Alshuth & Stockburger, 1986)]. Hanamoto et al. previously interpreted that the fast rising M component came from a second species of bR which was in dynamic equilibrium with bR_{570} (Hanamoto et al., 1984). Their interpretation is equivalent to our proposal of the thermal backreaction bR_{570} $\rightarrow$ N; their observation of a larger amplitude of the fast rising component at a higher pH is also consistent with our scheme, because light pulses at a high repetition rate (10 Hz) used in their experiment could accumulate a higher concentration of N at a higher pH.

Isomerizations of the Retinal Chromophore. Resonance Raman results and extraction experiments show that the retinal chromophore configuration is 13-trans in bR_{570} and O_{640} and 13-cis in L_{550} and M_{412} (Pettei et al., 1977; Hurley et al., 1978; Smith et al., 1985). Both Schulten and Mathies, in their photocycle models, require an N intermediate with a protonated 13-cis chromophore before the thermal isomerization to the 13-trans chromophore of O_{640} takes place (Schulten et al., 1984; Smith et al., 1986). Like the protonated 13-cis chromophore of dark-adapted bR or L_{550}, one would expect this N chromophore to absorb maximally between 550 and 560 nm. As pointed out above, evidence for the existence of such a chromophore was originally seen in the transient kinetics of pm suspensions (Lozier et al., 1975) and placed between M_{412} and O_{640}. It was also detected in experiments with pm multilayers which showed that it is photoactive and generates a transient photovoltage opposite to the proton-pumping direction (Hwang et al., 1978). One might then assume that excitation isomerizes the 13-cis chromophore and could generate a 13-trans chromophore which, via ^{N}L and ^{N}M, decays back to the 13-trans chromophore of bR_{570}. Consistent with this assumption would be the observation by Maeda et al. that at high pH resonance Raman spectra show an L-like intermediate with an all-trans chromophore, which they suggested could be the photoproduct of an earlier intermediate (Maeda et al., 1986).

Spectroscopically, the chromophore of N, at least so far, cannot be clearly distinguished from the 13-cis chromophore of dark-adapted bR, except for the generation of a slowly decaying M-like photoproduct and, perhaps, an L-like photoproduct with an all-trans chromophore. Reevaluation of earlier spectroscopic observations and chromophore extraction data on light- and dark-adapted bR is, therefore, required. For instance, Casadio et al. reported reduced light adaptation in Triton-solubilized monomeric bR (Casadio et al., 1980), but Drachev et al. showed that Triton X-100 promoted formation of N (P, R_{350}) (Drachev et al., 1986). Also, a reduced extent of light adaptation, or "dark adaptation by light", at wavelengths above 600 nm has been reported for monomeric bR in lipid vesicles and partly dehydrated multilayers of pm (Kouyama et al., 1985; Casadio & Stoeckenius, 1980; Korenstein & Hess, 1977, 1978). In all these conditions, slowly decaying M intermediates have been observed, and accumulation of N may have occurred. This is especially clearly shown in the high-pH spectra of Muccio and Cassim (1979). Similarly, a reevaluation of all investigations on M, where it was accumulated with low temperature and high pH, is required. Recent FTIR spectra have already indicated a difference between room-temperature and low-temperature M conformations (Braiman et al., 1987).

Proton Release and Uptake. We have shown here that the thermal relaxation $N \rightarrow bR_{570}$ and the transition $^{N}M \rightarrow bR_{570}$ are accompanied by proton uptake from the medium. Recently, Grzesiek and Dencher (1986) reported that one proton is released into the medium during the formation of M_{412} and taken up during its decay. At their experimental conditions, the bR photoreaction follows the cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightarrow O_{640} \rightarrow bR_{570}$, and the lifetime of N is comparable to or shorter than its rise time. Therefore, the present proposal of a proton-uptake process during the transition $N \rightarrow O_{640} \rightarrow bR_{570}$ (not during the transition $M_{412} \rightarrow N$) is not inconsistent with their observation that the rate of proton uptake is comparable to the rate of M_{412} decay. Since N almost certainly has a reprotonated Schiff base, this implies reprotonation from a group inside the membrane, which is later reprotonated from the cytoplasmic side medium, and the decay rate of N may be limited by the reprotonation rate of this group.

The present scheme provides a simple explanation for the earlier observations on the $[H^+]/[M_{412}]$ stoichiometry which has often been used to measure the number of protons transported in one bR turnover. Under stationary illumination, the ratio became much larger than 1.0 at high pH, at high ionic strength, and at low light intensity (Kuschmitz & Hess, 1981). Under these conditions, the amount of N accumulated in the light becomes much larger than the amount of M_{412}, and to determine the amount of H^+ released per bR cycling, the stoichiometry $[H^+]/([^{N}M] + [N] + [M_{412}])$ should be measured. The relation of the two components of the M decay and the H^+/M stoichiometry observed by these authors is also readily explained by our model. So are the H^+/M stoichiometries obtained by Govindjee et al. and the salt effect they observed for pm sheets (Govindjee et al., 1980). That they saw no salt effects in lipid vesicles is probably due to the much lower negative surface charge on the vesicles. However, the still unexplored effects of the electrochemical gradients may also have to be taken into account.

The recent study of light-induced pH changes in *H. halobium* envelope vesicle suspension in the presence of a high concentration of Mg^{2+} or Mn^{2+} has shown that bR translocates protons under continuous illumination, even at high external pH (pH 9.5) (Kouyama et al., 1987). On the basis of the analysis of flash-induced pH changes, on the other hand, Li et al. (1986) suggested that the apparent activity of the proton pump dropped dramatically above pH 8. The discrepancy between the two results may be explained by the different illumination conditions. At high pH and at high ionic strength, the cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightsquigarrow {}^{N}M \rightarrow bR_{570}$ becomes the dominant photoreaction under stationary illumination, whereas the cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightarrow bR_{570}$ would be the dominant reaction in the flash experiment. Only the former cycle would be effective for proton pumping at high pH, because of the slow thermal decay of N. This means that *H. halobium* can partly overcome the detrimental effect of high pH on its energy supply by using a second photon to complete the photoreaction cycle more efficiently.

In the proposed model (see Figure 9) we have incorporated speculative features of the proton transport, because they, like the rest of the model, should be of heuristic value. Recent advances in vibrational spectroscopy (Mathies et al., 1987; Engelhard et al., 1985; Brajman et al., 1987) should eventually allow identification of the groups involved and determination of differences in the chromophore conformation of L_{550}, M_{410}, N, ^{N}L, and ^{N}M, which are not readily distinguished by their visible absorption spectra.

Added in Proof

An intermediate with the expected properties of N, i.e., a 13-14-cis, C=N-anti, protonated Schiff base occurring after M_{412}, has recently been observed by resonance Raman spectroscopy (Fodor et al., 1988).

Acknowledgments

We thank Drs. K. Kinosita, A. Maeda, K. Ohno, J. F. Nagle, and R. A. Bogomolni and the members of the San Francisco group for fruitful discussions.

Registry No. H^+, 12408-02-5.

References

Alshuth, T., & Stockburger, M. (1986) *Photochem. Photobiol. 43*, 55–66.

Bogomolni, R. A., Baker, R. A., Lozier, R. H., & Stoeckenius, W. (1980) *Biochemistry 19*, 2152–2159.

Braiman, M. S., Ahl, P. L., & Rothschild, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5221–5225.

Casadio, R., & Stoeckenius, W. (1980) *Biochemistry 19*, 3374–3381.

Casadio, R., Gutowitz, H., Mowery, P., Taylor, M., & Stoeckenius, W. (1980) *Biochim. Biophys. Acta 590*, 13–23.

Dancshazy, Z., Govindjee, R., Nelson, B., & Ebrey, T. G. (1986) *FEBS Lett. 209*, 44–48.

Drachev, L. A., Kaulen, A. D., Skulachev, V. P., & Zorina, V. V. (1986) *FEBS Lett. 209*, 316–320.

Eisenbach, M., Bakker, E. P., Korenstein, R., & Caplan, S. R. (1976) *FEBS Lett. 71*, 228–232.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Fodor, S. P. A., Ames, J. B., Gebhard, R., van den Berg, E. M. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. A. (1988) *Biochemistry* (in press).

Gillbro, T., & Kriebel, A. N. (1977) *FEBS Lett. 79*, 29–32.

Gillbro, T., & Sundstrom, V. (1983) *Photochem. Photobiol. 37*, 445–455.

Gillbro, T., Kriebel, A. N., & Wild, U. P. (1977) *FEBS Lett. 78*, 57–60.

Govindjee, R., Ebrey, T. G., & Crofts, A. R. (1980) *Biophys. J. 30*, 231–242.

Groma, G. I., & Dancshazy, Z. S. (1986) *Biophys. J. 50*, 357–366.
Grzesiek, S., & Dencher, N. A. (1986) *FEBS Lett. 208*, 337–342.
Hanamoto, J. H., Dupuis, P., & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7083–7087.
Hess, B., & Kuschmitz, D. (1977) *FEBS Lett. 74*, 20–24.
Hurley, J. B., Becher, B., & Ebrey, T. G. (1978) *Nature (London) 272*, 87–88.
Hwang, S. B., Korenbrot, J. I., & Stoeckenius, W. (1978) *Biochim. Biophys. Acta 509*, 300–317.
Korenstein, R., & Hess, B. (1977) *Nature (London) 270*, 184–186.
Korenstein, R., & Hess, B. (1978) *FEBS Lett. 89*, 15–20.
Korenstein, R., Hess, B., & Markus, M. (1979) *FEBS Lett. 102*, 155–161.
Kouyama, T., Bogomolni, R. A., & Stoeckenius, W. (1985) *Biophys. J. 48*, 201–208.
Kouyama, T., Kouyama, A. N., & Ikegami, A. (1987) *Biophys. J. 51*, 839–841.
Kuschmitz, D., & Hess, B. (1981) *Biochemistry 21*, 5950–5957.
Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462–4466.
Li, Q., Govindjee, R., & Ebrye, T. G. (1986) *Photochem. Photobiol. 44*, 515–518.
Lozier, R. H., & Niederberger, W. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 1805–1809.
Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophys. J. 15*, 955–962.
Maeda, A., Ogura, T., & Kitagawa, T. (1986) *Biochemistry 25*, 2798–2803.
Mathew, M. K., Helgerson, S. L., Bivin, D., & Stoeckenius, W. (1985) *Biophys. J. 47*, 323a.
Mathies, R. A., Smith, S. O., & Palings, I. (1987) in *Biological Applications of Raman Spectroscopy*, Vol. 2, Wiley, New York.
Muccio, D. D., & Cassim, J. Y. (1979) *J. Mol. Biol. 135*, 595–609.
Nagle, J. F., Parodi, L. A., & Lozier, R. H. (1982) *Biophys. J. 38*, 161–174.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Oesterhelt, D., Hegemann, P., & Tittor, J. (1985) *EMBO J. 4*, 2351–2356.
Ohno, K., Takeuchi, Y., & Yoshida, M. (1981) *Photochem. Photobiol. 33*, 573–578.
Ort, D. R., & Parson, W. W. (1978) *J. Biol. Chem. 253*, 6158–6164.
Parodi, L. A., Lozier, R. H., Bhattacharjee, S. M., & Nagle, J. F. (1984) *Photochem. Photobiol. 40*, 501–512.
Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955–1959.
Scherrer, P., & Stoeckenius, W. (1985) *Biochemistry 24*, 7733–7740.
Schulten, K., Schulten, Z., & Tavan, P. (1984) in *Information and Energy Transduction in Biological Membranes*, Liss, New York.
Slifkin, M. A., & Caplan, S. R. (1975) *Nature (London) 253*, 56–58.
Smith, S. O., Lugtenburg, J., & Mathies, R. A. (1985) *J. Membr. Biol. 85*, 95–109.
Smith, S. O., Hornung, I., Van der Steen, R., Pardoen, J. A., Braiman, M. S., Lugtenburg, J., & Mathies, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 967–971.
Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215–278.
Tsuda, M., Glaccum, M., Nelson, B., & Ebrey, T. G. (1980) *Nature (London) 287*, 351–353.
Xie, A. H., Nagle, J. F., & Lozier, R. H. (1987) *Biophys. J. 51*, 627–635.

Interaction of Plastocyanin with Photosystem I: A Chemical Cross-Linking Study of the Polypeptide That Binds Plastocyanin†

R. Max Wynn and Richard Malkin*
Division of Molecular Plant Biology, 313 Hilgard Hall, University of California, Berkeley, California 94720
Received February 26, 1988

ABSTRACT: Plastocyanin has been covalently cross-linked to photosystem I (PSI) by using a water-soluble cross-linker, *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The cross-linking reaction is light stimulated and results in the disappearance of a single 19-kDa subunit of PSI with the formation of a new protein-staining component of 31 kDa. The new product at 31 kDa reacts with both plastocyanin and 19-kDa subunit antibodies. Carboxyl group modified plastocyanin does not form a cross-linked product with PSI, implying that the negatively charged surface-exposed groups on plastocyanin are necessary to stabilize binding. These results demonstrate a specific interaction of plastocyanin with PSI and further implicate a specific protein to which plastocyanin binds to facilitate electron transfer to the P700 reaction center.

Electron transport in oxygen-evolving membranes of cyanobacteria, algae, and higher plants occurs through the cooperative interaction of two photochemical systems which results in the reduction of NADP with water as the electron donor. Electrons are shuttled between these photosystems by a third membrane complex, the cytochrome b_6–f complex. The cytochrome complex mediates electron flow from photosystem II (PSII) to photosystem I (PSI)[1] through two soluble electron

† Supported in part by a grant from the National Science Foundation (DMB-8711206).
* To whom correspondence should be addressed.

0006-2960/88/0427-5863$01.50/0