Turnover Rate of the Proton Pumping Cycle of Bacteriorhodopsin: pH and Light-Intensity Dependences†

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ABSTRACT: The photoreaction and the proton pump activity of bacteriorhodopsin (bR) were investigated with acid-treated envelope vesicles of *Halobacterium halobium*. As long as a low light intensity was used so as not to accumulate any long-lived photoproduct, the initial rate of light-induced proton release from the envelope vesicles was constant from pH 3.5 to 9.0. Inhibition of the thermal relaxation of the trans photocycle intermediate N_{560} at an extremely alkaline pH was accompanied by a decrease in the proton pumping rate. At high light intensities, however, the proton pumping rate could be higher than the value that would be expected from the turnover rate of the one-photon cycle ($bR_{570} \rightsquigarrow M_{412} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{570}$). The relatively higher rate was explained by the two-photon cycle ($bR_{570} \rightsquigarrow M_{412} \rightarrow N_{560} \rightsquigarrow {}^{N}M \rightarrow bR_{570}$) that was proposed in a previous paper [Kouyama, T., Nasuda-Kouyama, A., Ikegami, A., Mathew, M. K., & Stoeckenius, W. (1988) *Biochemistry 27*, 5855–5863]. Light absorption by N_{560} does not have an inhibitory effect on the proton translocation across the membrane; it rather accelerates the completion of the proton pumping cycle. Even at physiological pH, the two-photon cycle became the dominant reaction when a large pH gradient was generated in the light. That is, the photoproduct ${}^{N}M$ was accumulated when the envelope vesicle suspension at neutral or weakly acidic pH was irradiated with strong light. The time lag between the onset of illumination and the accumulation of ${}^{N}M$ became longer as the external pH was made more acidic; i.e., increase of the internal pH above a critical value was required for the ${}^{N}M$ formation. It is suggested that the thermal relaxation of N_{560} is inhibited as the internal pH becomes high. Also, the ${}^{N}M$ decay was suggested to be regulated by the internal pH. The results obtained are in harmony with the scheme that both the light reaction $N_{560} \rightsquigarrow {}^{N}M \rightarrow bR_{570}$ and the thermal relaxation $N_{560} \rightarrow O_{640} \rightarrow bR_{570}$ are accompanied by proton uptake from the cytoplasmic side medium.

Bacteriorhodopsin (bR)[1] is a transmembrane protein that works as a light-driven proton pump. In the plasma membrane of *Halobacterium halobium*, bR molecules aggregate to form a two-dimensional crystalline patch, which is called purple membrane (pm). The color of purple membrane is due to a retinal chromophore that is bound to the ϵ-amino group of a lysine residue in the apoprotein via a protonated Schiff base linkage. When a bR isomer (bR_{570}) containing *all-trans*-retinal absorbs light, it undergoes a cyclic photoreaction that is accompanied by proton translocation across the membrane from the cytoplasmic side to the external medium. The proton gradient thus generated is utilized by the halobacteria for ATP synthesis and other cellular processes [for review, see Stoeckenius et al. (1979), Khorana (1988), Kouyama et al. (1988a), and Rothschild (1988)].

In an earlier model (Lozier et al., 1975, 1977), the photocycle of bR_{570} was described with five intermediates, K_{590}, L_{550}, M_{412}, N_{530}, and O_{640} (the subscripts indicate absorption maxima). A transient deprotonation of the Schiff base, isomerization around the C13–14 double bond, and protonation/deprotonation of amino acid residues such as aspartic acid and tyrosine have been shown to occur during the bR photocycle (Engelhard et al., 1985; Smith et al., 1986; Rothschild, 1988). Until recently, however, the N intermediate was forgotten, and, as a consequence, details of the bR photoreaction remained a matter of controversy [for review, see Nagle et al. (1982)]. Recent studies of subsecond absorption kinetics of pm at alkaline pH (Kouyama et al., 1988b) or in the presence of Triton X-100 (Drachev et al., 1986, 1987) have shown an intermediate with an absorption maximum near 560 nm that occurs after M_{412}. We shall refer to this intermediate as N_{560}. The difference absorption spectrum between N_{560} and bR_{570} is characterized by a large negative peak at 580 nm and small positive peaks in the near-UV region. An intermediate with an absorption peak at 350 nm, R_{350}, which was reported by Dancsházy et al. (1986), is probably identical with N_{560}. It has been shown that one proton is released into the medium during the formation of M_{412} (Grzesiek & Dencher, 1986) and taken up during the thermal relaxation $N_{560} \rightarrow bR_{570}$ (Drachev et al., 1986; Kouyama et al., 1988b). The thermal relaxation of N_{560}, which is fast at low pH, is inhibited by increasing pH. The chromophore in N_{560} is presumably reprotonated from a group inside the protein, which is, in turn, reprotonated from the medium when N_{560} decays. A recent resonance Raman study (Fodor et al., 1988) has shown that N_{560} has a reprotonated Schiff base and the configuration of C13–14 cis and C=N anti; i.e., reprotonation of the Schiff base precedes isomerization around the C13–14 double bond. To explain a unidirectional movement of protons during the bR photocycle, therefore, one needs to postulate some conformational change in the protein moiety.

The intermediate N_{560} is also photoactive (Hwang et al., 1978). From a comprehensive study of millisecond and subsecond absorption kinetics of an alkaline suspension of pm (Kouyama et al., 1988b), it has been shown that there exists a light-initiated pathway from N_{560} to bR_{570}. That is, excitation of N_{560} produces an M-like photoproduct, ${}^{N}M$, which

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[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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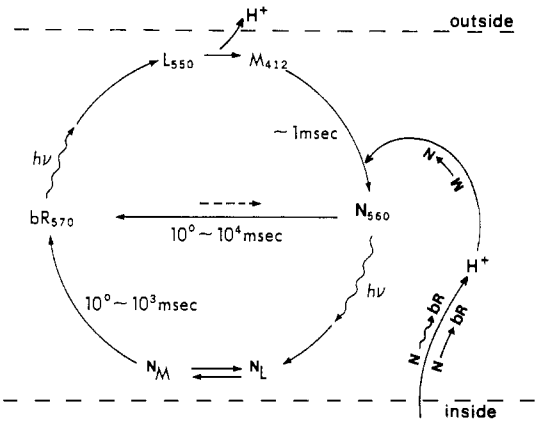

FIGURE 1: Model for the photoreaction cycle of light-adapted bR (Kouyama et al., 1988b). At neutral or acidic pH, N_{560} decays rapidly to O_{640} (not shown), which then decays to bR_{570} within 10 ms at room temperature. At high pH, the thermal decay of N_{560} is inhibited so that O_{640} is no longer detectable. The chromophore in N_{560} is presumably reprotonated from a group inside the protein, which, in turn, is reprotonated from the medium when N_{560} decays. When N_{560} absorbs light, it is converted to bR_{570} via a long-lived M-like photoproduct ^NM. At high light intensities, therefore, the bR photoreaction is approximated by the two-photon cycle: $bR_{570} \rightsquigarrow M_{412} \rightarrow N_{560} \rightsquigarrow {}^NM \rightarrow bR_{570}$.

decays to bR_{570} (Figure 1). When the thermal relaxation of N_{560} is inhibited at high pH, the overall photoreaction of bR in strong continuous light is approximated by the two-photon cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N_{560} \rightsquigarrow {}^NM \rightarrow bR_{570}$. The ^NM intermediate, which shows the absorption maximum at 410 nm, is characterized by its long lifetime at high pH; i.e., alkalization of the medium causes inhibition of the transition $^NM \rightarrow bR_{570}$, which is also accompanied by proton uptake from the medium. The problem to be clarified is, from which side of the membrane are protons taken up during the light reaction $N_{560} \rightsquigarrow {}^NM \rightarrow bR_{570}$? In other words, does the light-initiated reaction from N_{560} to bR_{570} have an inhibitory or promoting effect on the proton translocation?

In the presence study, our previous work on the importance of the N intermediate and the two-photon cycle in pm sheets (an open system; Kouyama et al., 1988b) was extended to *H. halobium* envelope vesicles (a closed system). Throughout this paper, we address the following hypotheses and questions: (1) the two-photon cycle concept; (2) the promotion of proton translocation by a second absorbed photon; (3) the change in the proton pumping quantum efficiency with pH versus the pH dependence of the turnover rate of the proton pumping cycle; (4) the effect of the internal pH on the proton uptake phase versus the effect of membrane potential on intramembrane proton-transfer processes.

Materials and Methods

Envelope vesicles of *Halobacterium halobium* JW3 were prepared as described earlier (Kouyama et al., 1987). The cells harvested from a steady-state culture were broken by the freeze–thaw method, and the resultant envelope vesicles were sedimented by centrifugation at 10000*g* for 30 min. The procedure of freeze–thaw and centrifugation was repeated three times in a basal salt solution and twice in 3 M KCl, and a reddish band at the top of the sediment and debris at the bottom were removed. For optical measurements, the envelope vesicles were layered on a linear density gradient (5%–22% Ficoll in 3 M KCl) and centrifuged at 10000*g*, and a broad purple band near the bottom, which contained a negligible amount of caroetnoid, was collected. The purified envelope vesicles contained two major proteins, bR and a ~200-kDa glycoprotein; other proteins, except a 85-kDa protein, were very minor. The diameter of the envelope vesicles was about 0.4 μM, as judged from the extent of extrusion through nuclepore membranes. Before measurements of light-induced pH or absorption change, the envelope vesicles were treated with acid (pH ~2.5) at room temperature for 10–20 min; thereafter, the membrane permeability to proton was greatly reduced. [The original sample at pH 6–7 exhibited a biphasic pH response upon illumination (acidification followed by alkalization of the medium), and the alkalization component disappeared after the acid treatment.] The bR content was determined by using the molar absorption coefficient of 63000 M^{-1} cm^{-1} at 568 nm (Rehorek & Heyn, 1979). Purple membrane fragments were prepared according to the established procedure (Oesterhelt & Stoeckenius, 1974).

Light-induced pH changes were measured with a glass pH electrode (Radiometer GK2321C, Copenhagen) connected to a Horiba pH meter M8s, as described earlier (Kouyama et al., 1987). The response time of the pH electrode was ~1 s.

Subsecond absorption kinetics were measured with a cross-illumination spectrophotometer (Kouyama et al., 1988a,b). Briefly, measuring light and actinic light were alternately separated with mechanical choppers at a frequency of ~200 Hz so that absorption spectra were recorded in the presence of intense actinic light. Actinic light from an Xe lamp (300 W) was passed through a heat-absorbing water filter, reflected from a cold mirror (400–700 nm), passed through an optical filter (>540 nm), and then focused into a sample cell. For measurements of the quantum efficiency of the photoreaction of N_{560}, emission from the Xe lamp was divided into two beams, which were then passed through independent optical shutters and again combined so as to irradiate the sample cell in parallel with each other. 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 (Hamamatsu R374). Its photocurrent was amplified under the supervision of gating pulses from the choppers, digitized, and stored in the memory of a personal computer (NEC PC9800/VM2).

Millisecond absorption kinetics measurements were performed with a constant-power Xe flash lamp (Nissin Electronic Co., Ltd., Tokyo) providing light pulses of 10 μs width (Kouyama et al., 1988b). Light pulses, above 540 nm and at the frequency of 0.25 Hz, irradiated the sample cell at a right angle to measuring light, and each light pulse excited a few percent of the pigment. The amplified photosignal was fed to a digital memory scope (Iwatsu DS6121, Tokyo) operated in a pretrigger mode. For flash-photolysis experiments in the presence of background light, emission from a 300-W projector lamp was passed through optical filters (540–660 nm) and reflected into the sample cell by a half-mirror that was set in the optical path of the pulsed light.

Results

Light-Induced Proton Release from H. halobium Envelope Vesicles. Light-induced pH changes in the envelope vesicle suspension in 3 M KCl were investigated at different external pH values. In the following experiments, the pH changes were followed with the glass pH electrode, and a low concentration of pH buffering molecules (2 mM HEPES and 2 mM MES but no divalent cations) were added so that light-induced pH changes in the external medium were restricted within 0.2 pH unit. (In unbuffered systems, the light-induced pH change at pH ~8 could be as large as 1 pH unit.) When yellow light

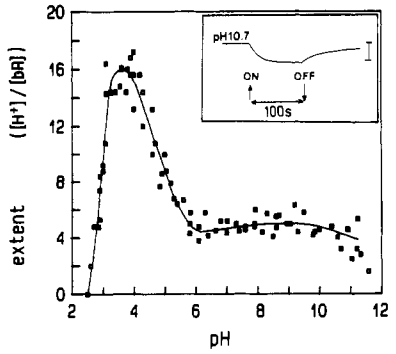

FIGURE 2: Number of protons released from *H. halobium* envelope vesicles during the first 100 s of illumination plotted as a function of the external pH. The envelope vesicles containing 25 nmol of bR were suspended in ~1.2 mL of 3 M KCl solution (+2 mM HEPES and 2 mM MES), and the suspension in a vial cuvette (1-cm diameter) with a magnetic stirrer at 5 °C was irradiated by yellow light (500–660 nm) at 40 mW/cm^2. The external pH reached a minimum value within 100 s after the light was turned on, except at extremely acidic pH (pH 3.5–2.5). (Insert) Example of light-induced pH changes in the external medium. The bar on the right-hand side indicates the pH change induced by a pulse of 100 nmol of HCl.

(500–660 nm) at 40 mW/cm^2 was used, the external pH reached a minimal value within 50 s after the light was turned on, except in an extremely acidic pH region (pH <3.5) where light-induced pH decreases lasted a few minutes or longer (not shown). In Figure 2, the extent of proton release observed during the first 100-s illumination is plotted as a function of the external pH. Since the extent of proton release is dependent on the buffering capacity of the internal space of the envelope vesicle, this quantity cannot be used as a direct measure of the proton pumping activity of bR. Nonetheless, the result obtained clearly indicates that bR is capable of translocating protons in a wide pH range. That is, a noticeable number of protons were released even at very high pH values. These protons were suggested to reflect the actual translocation of protons across the membrane, because a pm suspension under the same solvent condition exhibited a much smaller pH change in the light.

At extremely alkaline pH values (pH >11.5) the envelope vesicle became unstable, as judged from an irreversible decrease in the magnitude of light-induced pH change. Thus, it was difficult to quantitatively determine the upper limit of the external pH for an active proton translocation. At acidic pH values, on the other hand, the envelope vesicle was tolerably stable, and the limiting pH for an active proton translocation was shown to be pH 2.5.

In Figure 3, the initial rates of light-induced proton release observed at different light intensities are plotted as a function of the external pH. The term "initial rate" used hereafter indicates an experimental quantity determined from the initial part (a few seconds) of light-induced pH change, in which the pH change was apparently linear with time. At a low light intensity (3 mW/cm^2), the initial rate was independent of the external pH from pH 3.5 to 9. At a much higher light intensity (40 mW/cm^2), however, the initial rate of proton release came to depend on the external pH; i.e., it was high at a weakly acidic pH and decreased gradually at alkaline pH. In other words, deviation from a linear dependence of the initial rate on the light intensity was more significant at a high pH. Temperature of the vesicle suspension was another factor affecting the proton pumping rate. When only the profile of the pH dependence of the initial rate was considered, the decrease of temperature had the same effect as the increase of light intensity; compare the data obtained at 3 mW/cm^2 and 5 °C with those obtained at 40 mW/cm^2 and 25 °C.

In relation to the pH and temperature dependences of the initial rate of proton release, the turnover of the bR photocycle slowed down at higher pH or at lower temperature. In Figure 4, the results of the absorption kinetics of a pm suspension in 3 M KCl are summarized. At 5 °C, the lifetime of the N_{560} intermediate increased from 200 ms to 200 s between pH 8 and 11.5. On the other hand, even at the lowest light intensity (3 mW/cm^2) used in the measurements of light-induced pH change, each bR molecule was calculated to be excited every few seconds. Under the illumination condition that each bR molecule could be hit by several photons before the completion of its photocycle, the quantum efficiency of light-induced proton release was expected to be low. Thus, we should have used a much lower light intensity to correctly evaluate the proton pumping quantum efficiency in the extremely alkaline region, where the lifetime of N_{560} increased exponentially with the increase of pH. It seems possible that, at an infinitely low light intensity, the proton pumping quantum efficiency is

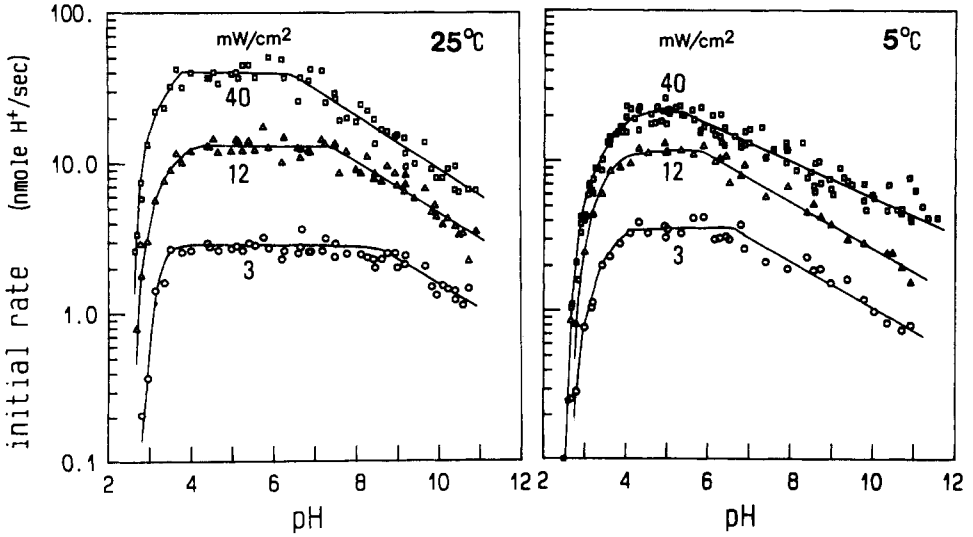

FIGURE 3: Initial rate of light-induced proton release from the envelope vesicles. The vesicle suspension at 5 (right) or 25 °C (left) was irradiated with yellow light (500–660 nm) at the intensity of 40 (□), 12 (△), or 3 mW/cm^2 (○). The sample containing 20 nmol of bR was irradiated for 10–30 s and kept in the dark for 5–10 min before the next illumination. The response time of the pH electrode was ~1 s, and the rate constants shown here were calculated from initial parts (2–5 s) of light-induced pH change in which the pH change was apparently linear with respect to time.

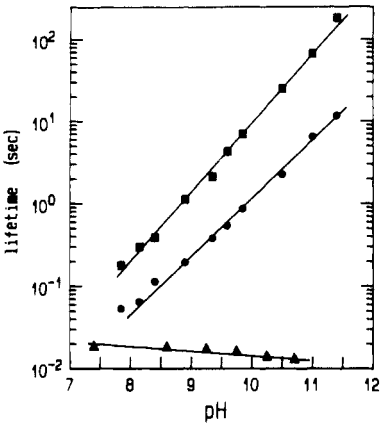

FIGURE 4: Lifetimes of N_{560} (■), ^{N}M (●), and M_{412} (▲) plotted as a function of the pH of the medium of a pm suspension in 3 M KCl (+10 mM bicarbonate) at 5 °C. The lifetimes at 25 °C were shorter by a factor of 5–10. The lifetimes were calculated by the same procedure as described previously (Kouyama et al., 1988b). Briefly, the lifetime of N_{560} was determined from the subsecond absorption kinetics at 580 nm after weak actinic light (540–700 nm, <8 mW/cm^2) was turned off, the lifetime of ^{N}M from the absorption recovery at 410 nm after strong actinic light (30 mW/cm^2) was turned off, and the lifetime of M_{412} from the flash-induced absorption kinetics at 410 nm.

constant over the entire pH region in which bR_{570} is stable.

Slowdown of the turnover of the bR photoreaction can be one reason for the decrease in the proton pumping rate at high pH. But there was no guarantee that bR molecules in the cell envelope vesicle underwent the same photoreaction as in pm fragments. It seems reasonable to assume that the bR photoreaction is affected by a proton gradient across the membrane. In the following paragraphs, the effect of a light-induced pH gradient on the millisecond and subsecond absorption kinetics of bR will be presented. For a better interpretation of absorption kinetics data, it would be desirable to simultaneously monitor the light-induced pH gradient. Unfortunately, the fluorescence method, which has been used to monitor the internal pH of reconstituted vesicles (Seigneuret & Rigaud, 1983), could not be applied to the present system, because the membrane of the envelope vesicles was significantly permeable to fluorescent indicators like pyranine ($\tau \sim$ 1 h). Instead, we measured the buffering capacity of the vesicle interior so as to estimate the magnitude of the internal pH change from the number of protons released in the light. That is, the pH titration of an unbuffered suspension of the envelope vesicles was carried out in the absence and presence of 0.05% Triton X-100 (Figure 5). The result indicates that, at alkaline pH, the buffering capacity of the vesicle interior is very low; i.e., extraction of one proton per bR from the vesicle interior would result in an increase by an order of 1 pH unit in the internal pH. A low concentration of buffer molecules (2 mM HEPES and MES) added in measurements of light-induced pH change were calculated to only slihtly increase the internal buffering capacity. Figure 2 thus suggests that the pH gradient generated in the strong light may reach 4 pH units, the maximal value for light-induced pH gradients so far reported (Kouyama et al., 1988a).

Light-Induced Absorption Changes in the Envelope Vesicle. When the envelope vesicles were exposed to high light intensities, the turnover of the bR photocycle became significantly slower with the increase of the illumination period. The inserted curves in Figure 6 show light-induced absorption changes in the light-adapted suspension of the envelope vesicles at pH 6.6. When the suspension was irradiated with orange light (30 mW/cm^2, 540–700 nm), a quick decrease in the

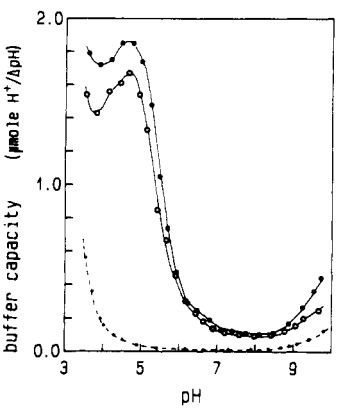

FIGURE 5: pH titration of the envelope vesicle suspension containing 31 nmol of bR in the absence (○) and presence (●) of 0.05% Triton X-100, i.e., the inverse of the pH change induced by a HCl pulse plotted against the pH of the medium. The detergent made the membrane very leaky to proton so that the light-induced pH change became very small (<0.001 pH unit). The difference between the two curves came primarily from the buffering action of the vesicle interior that was not accessible in the absence of the detergent, provided that the detergent did not disturb the protein–lipid interaction. The glycoprotein of the surface layer (Lechner & Sumper, 1987), which was not removed during the preparation procedure of the envelope vesicle, explains the strong buffering action of the vesicle outside. The broken line shows the buffering capacity of the medium alone (1 mL, 3 M KCl).

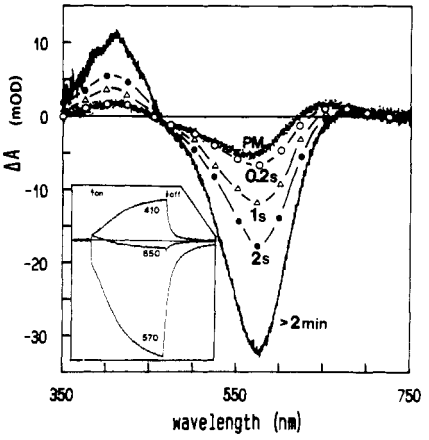

FIGURE 6: Light-induced absorption changes in the *H. halobium* envelope vesicle suspension at pH 6.4. The amplitudes of light-induced absorption change observed 0.2 (○), 1 (△), and 2 s (●) after orange light (540–700 nm; 30 mW/cm^2) was turned on are plotted as a function of the measuring wavelength. These amplitudes were calculated from the absorption changes observed when a preilluminated suspension (OD_{570} = 0.27) was irradiated by orange light for 4 s every 110 s; examples are the inserted curves, which were obtained by averaging 10 illuminations. The solid line shows the light-induced difference absorption spectrum that was observed when the envelope vesicles were irradiated for >2 min; the dotted line represents the corresponding spectrum obtained when a pm suspension with the same bR content was irradiated. Solvent: 3 M KCl, 2 mM MES, 2 mM HEPES, at 25 °C.

absorbance at 570 nm was followed by a slow decrease that lasted more than 10 s. A similar but inverted absorption change was observed at 410 nm. At 650 nm, the absorbance first increased and then decreased. When the illumination was terminated, the absorbance at 570 or 410 nm slowly returned to the level observed before the illumination. The half-time of the absorption recovery became longer with increasing illumination time, and the time constant of the slowest component was as long as a few seconds at room temperature. When the vesicle suspension was irradiated for a very short period (~50 ms), on the other hand, the absorption recovery at 410 nm completed within 20 ms. In a pm suspension at

neutral or acidic pH, the absorption recovery was always fast and its kinetics did not depend on the duration of illumination.

In the envelope vesicle suspension, the profile of light-induced difference absorption spectra changed with the illumination period (Figure 6). At the beginning of illumination, small absorption increases at 410 and 650 nm were induced. These small positive peaks, which were also seen in pm fragments under the same solvent condition (the dotted line in Figure 6), are presumably due to accumulation of M_{412} and O_{640} (Lozier et al., 1975). The absorption decrease at 580 nm was more significant in the envelope vesicles than in pm fragments, however. Since the difference spectrum between N_{560} and bR_{570} has a maximal amplitude at 580 nm (Kouyama et al., 1988b), the observed absorption changes suggested that the thermal decay of N_{560} was inhibited in the envelope vesicles. When the duration of illumination was further increased, the envelope vesicle suspension exhibited a large increase of the 410-nm band that was accompanied by depletion of the visible band. The light-induced difference spectrum observed in the envelope vesicles irradiated for a prolonged period (>2 min) was rather similar to that observed in an alkaline suspension of pm (at pH 9). In the latter suspension, the accumulation of an M-like photoproduct of N_{560}, ^{N}M, is responsible for the large increase in the 410-nm absorbance (Kouyama et al., 1988b). When the vesicle suspension was irradiated at a lower light intensity (2 mW/cm^2, 540–700 nm), the absorption change induced by long illumination was characterized by a relatively large change at 580 nm; no absorption increase was detected in the long-wavelength region, and the absorption increase at 410 nm was small. When an uncoupler CCCP was added, the light-induced difference absorption spectrum of the envelope vesicles was not different from that observed in pm sheets under the same solvent condition. We suggest that, as the light-induced proton gradient becomes larger, the thermal decay of N_{560} is first inhibited and then its photoproduct ^{N}M is generated. [It will be shown later that the lifetime (~1 ms) of M_{412} is little affected by a light-induced pH gradient.] The observation of a slower absorption recovery at 570 nm than at 410 nm (the inserted curves in Figure 6) is indicative of a longer lifetime of N_{560} than ^{N}M; this tendency was also seen in an alkaline suspension of pm (Figure 4).

Even when the envelope vesicles were suspended at acidic pH, a large increase at 410 nm was induced by strong light. At lower pH, however, longer illumination was required before a large absorption increase at 410 nm was observed (the inserted curves in Figure 7). That is, the absorption kinetics after the light was turned on were described with two phases (a fast increasing phase followed by a slowly increasing phase), and the time lag between the two phases became longer as the medium became more acidic. At pH 4, the time lag was several seconds. In Figure 7, the absorption changes for different illumination times are plotted against the external pH and compared with those observed in pm suspensions (the solid circles). When the illumination period was short (e.g., 0.5 s), there was no large difference in the absorption change for the topologically closed system (vesicle) and the pm sheets, except at weakly alkaline pH. But, with increasing illumination time, a significant difference between the two systems appeared and extended toward the acidic region. Apparently, whenever the internal pH increases above a critical value (~pH 9), the thermal decays of N_{560} and ^{N}M are inhibited.

The absorption kinetics of the envelope vesicles in the presence of divalent cations also suggested strong dependence of the bR photoreaction on the internal pH. That is, at a high concentration (80 mM) of Mn^{2+}, which acts as a strong buffer

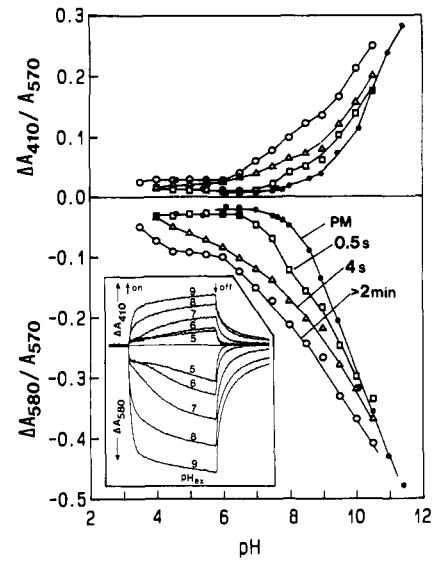

FIGURE 7: Magnitudes of absorption changes observed when the envelope vesicles were irradiated for 0.5 s (□), 4 s (△), and >2 min (○) are plotted as a function of the external pH. Below pH 4.5, dark adaptation of bR took place so fast that its contribution to the absorption change was not excluded. The solid circles represent the data obtained when a pm suspension with the same bR content was irradiated for >2 min. (pm fragments tended to aggregate below pH 6; the magnitude of light-induced absorption change became larger whenever there appeared a large aggregation.) Solvent: 3 M KCl, 2 mM MES, 2 mM HEPES, at 25 °C. (Insert) Light-induced absorption changes in the envelope vesicle suspension at different pH values. The illumination condition was the same as shown in Figure 6.

against the increase of the internal pH above pH 8.5, no large bleaching of the visible absorption band was induced by 4-s illumination. We also examined an alkaline suspension of the envelope vesicles in the presence of 80 mM Mg^{2+}, in which a large decrease in the external pH can be induced by long illumination (Kouyama et al., 1987). It was then found that the extent of light-induced depletion of the visible band became rather smaller as the external pH, which was monitored with pyranine, decreased from pH >8 to pH ~6 in strong continuous light (30 mW/cm^2). This result suggests that the pH gradient itself is not responsible for the accumulation of N_{560} and ^{N}M.

Flash-Induced Absorption Changes in the Envelope Vesicle Suspension. It has been shown that the millisecond absorption kinetics of an alkaline suspension of pm are greatly affected by background light; i.e., the amplitude of the slow decay component in the 410-nm absorption transient was shown to increase in proportion to the concentration of N_{560} accumulated in background light, and the fast (~2 ms at 25 °C) and the slow (10–1000 ms) decay components were attributed to $M_{412} \rightarrow N_{560}$ and $^{N}M \rightarrow bR_{570}$, respectively (Kouyama et al., 1988b). We found in the present study, even at neutral pH, a slowly decaying component(s) in the 410-nm absorption transient of the illuminated envelope vesicle (not shown). Its time constant depended on the intensity of background light [at 4 mW/cm^2 (580–660 nm), $\tau_s \sim 80$ ms], though the time constant of the fast-decay component was scarcely affected ($\tau_f = 2$ ms). In the absence of background light, the observed absorption kinetics were not different from those seen in a pm suspension with the same medium.

The result is substantially the same as reported previously by Groma et al. (1984) and indicated as a feedback effect of the light-induced membrane potential. In the present system, however, the internal pH was allowed to freely increase in the light, and a large amount of ^{N}M was shown to accumulate in

the illuminated envelope vesicles (Figure 6). Therefore, we attribute the slow-decay component to the decay process of ^{N}M.

Discussion

pH Dependence of the Proton Pump Activity of bR. The present result shows that, as long as the light intensity is low, the initial rate of light-induced proton release from the envelope vesicles does not depend on pH, at least in the pH range 4–9. A sharp decrease in the proton pumping rate seen below pH 3.5 can be readily explained by the acidic transition of bR (Mowery et al., 1979; Fischer et al., 1979). Since similar observations have been reported repeatedly (Drachev et al., 1981), we will not add any more, except for the observation of a strong correlation between the quantum efficiency of M_{412} formation and the proton pumping rate. (In the envelope vesicles, a color change due to the acidic transition took place around pH 2.8). The following discussion focuses on the proton pump activity in the alkaline pH region, because it has been a matter of controversy.

Ort and Parson (1979) investigated flash-induced proton release from pm fragments and found little variation in the proton pumping quantum efficiency in the pH range 6–8.75. Ormos et al. (1985) reported that the photocurrent in an oriented sample of pm fragments is independent of pH in the pH range 4–10. Our result is in harmony with these results. (For quantitative comparisons we need to take the salt concentrations at the different experiments into account, because at low salt concentration the effective pH ranges are different from the ones measured in the bulk.) A strong pH dependence of the proton pump activity was reported by several workers, however. Renard and Delmelle (1980) investigated the initial rate of light-induced proton release from *H. halobium* cells pretreated with DCCD, and they concluded that the proton pumping quantum efficiency was highest (0.6) at acidic pH, which decreased to a lower value (0.3) at pH 8–9. Interestingly, their experimental data resemble those shown by the open squares in the left panel of Figure 3, which were obtained at high light intensity. It has recently been shown that in an alkaline suspension of pm fragments the lifetime of the N_{560} intermediate increases exponentially with the pH of the medium (Kouyama et al., 1988b). As judged from the illumination condition used by Renard and Delmelle (500-W Hg lamp), it is likely that their data at alkaline pH were obtained under a condition at which a large fraction of photons were absorbed by N_{560}. Under this condition the proton pumping quantum efficiency will decrease, unless excitation of N_{560} is accompanied by translocation of a second proton. The effect of this photoreaction is expected to emerge more significantly at higher light intensity, at higher pH, and at lower temperature, and this tendency was experimentally observed (Figure 3).

To explain all of the data in Figure 3, we need to take account of many other factors. One is a nonnegligible contribution of the two-photon cycle ($bR_{570} \rightsquigarrow M_{412} \rightarrow N_{560} \rightsquigarrow {}^{N}M \rightarrow bR_{570}$) to the proton pumping activity, especialy at very high pH, which will be discussed below in more detail. Another factor is the accumulation of photoproducts like ^{N}M at high pH (Figure 7), because the data shown in Figure 3 were not normalized with respect to the concentration of the ground state of bR. Also, a rapid increase in the internal pH should be considered. In the pH region (pH ~8) where the buffering capacity of the internal space is lowest [a value much less than 3 nmol of H^+ (nmol of bR)$^{-1}$ (Δ pH)$^{-1}$; Figure 5], a pH change of more than 1 pH unit may be induced within 1 s after strong light is turned on. On the other hand, the initial rate of proton release was experimentally determined from the initial part (2–5 s) of light-induced pH change in which the pH change was apparently linear with time. Thus, the data obtained at high light intensities reflect the proton pumping activity in the presence of a considerably large pH gradient. The slow response of the pH electrode seems to explain why the data obtained at high light intensities exhibited a significant pH dependence even near neutral pH. As long as a glass electrode is used, it may be impossible to evaluate the true initial rate at high light intensities. (Fast pH changes can be followed with pH indicators but, in this case, it may be more difficult to distinguish the protons actually translocated from the protons transiently released or taken up during the bR photocycle.) Light-induced membrane potential may also pertain, but its effect was not elucidated in the present system in which valinomycin, which is usually used to relax the membrane potential, did not work well.

A sharp decrease in the proton pumping quantum efficiency above pH 7 is seen in the data published by Li et al. (1986). In their experiments, cell envelope vesicles were illuminated by flash light with a half-pulse width of 200 ms. Excitation of any photointermediate may be negligible when its lifetime is much shorter than the pulse width, but the multiphoton excitation problem becomes serious when the lifetime of N_{560} is comparable to or longer than the pulse width. The existence of N_{560} was not considered in their analysis. The same authors (Li et al., 1984) also investigated flash-induced proton release from DCCD-treated cells with actinic light with a much shorter pulse width (0.1 ms) and found a slight decrease in the proton pumping quantum efficiency at alkaline pH. In this case, the pulse width is shorter than the rise time of N_{560} (a few milliseconds), and the pH-dependent lifetime of N_{560} need not be considered. Previously, Dancsházy et al. (1983) observed that flash-induced absorption kinetics of intact cells were dependent on the light intensity. For a better interpretation of the data of flash-induced proton release from a topologically closed membrane vesicle, we need to explore the feedback effect of the electric potential that would be transiently generated by a synchronous movement of numerous protons. Also, it is necessary to elucidate the properties of other membrane proteins like Na^+/H^+ antiporter. In fact, Li et al. (1986) observed that the cell envelope membrane became permeable to protons at pH higher than 8. In this relation, Michel and Oesterhelt (1980) reported that in DCCD-treated cells the membrane potential generated in the light was low at pH 6 and high at pH 8, whereas the pH gradient was large at pH 6 and small at pH 8. It seems likely that some membrane proteins mediate an efficient conversion between the membrane potential and the pH gradient and their activity is dependent on the pH. The action of interfering membrane proteins was less significant in our sample, because the envelope vesicle prepared from the strain JW3, which is known to overproduce bR, contained a high density of bR (>70% of the total membrane protein).

Turnover Rate of the Proton Pumping Cycle at High pH. The photoreaction scheme shown in Figure 1 predicts that, when the thermal relaxation of N_{560} is inhibited, the turnover rate of the proton pumping cycle can be increased by excitation of N_{560}. In accordance with this prediction, the rate of light-induced proton release always increased with the light intensity; this was the case even when a large amount of N_{560} had been accumulated. This result indicates that excitation of N_{560} does not have an inhibitory effect on the proton translocation. We observed that, at extremely alkaline pH and at high light intensities, the rate of proton release from

the envelope vesicles was much faster than would be expected from the thermal decay rate of N_{560}. For instance, at 5 °C and pH 11, about four protons per bR were released within 100 s after the onset of steady-state ilumination (Figure 2), whereas N_{560} thermally relaxed with a time constant of 100 s (Figure 4). It is different to explain the relatively fast proton release by the "classic" photoreaction scheme of bR, in which only the one-photon cycle is considered, because it has been shown that only one proton is translocated during the one-photon cycle (Drachev et al., 1984; Grzesiek & Dencher, 1988).

On the basis of the above observation, we conclude that the two-photon cycle also effectively translocates protons. In fact, the proton pumping rate observed at high pH and at high light intensity can be explained if one proton is translocated during the two-photon cycle. At pH 11 and at 5 °C, the proton pumping rate observed at the highest light intensity was 0.08–0.2 proton bR^{-1} s^{-1}. (The higher value represents the initial rate of proton release, and the lower value was determined from the illumination time required for the release of two protons per bR). These values are not much different from the turnover rate (0.05–0.18 s^{-1}) of the two-photon cycle, in which the transition $^{N}M \rightarrow bR_{570}$ becomes the rate-limiting step. (The higher value was calculated from the lifetime of ^{N}M in pm sheets and the lower value from the absorption recovery at 410 nm after 30 s of illumination of the envelope vesicles with orange light at 30 mW/cm².) In the above comparison we assumed the following equation: $d[H^+]/dt \sim k_{NM}[^{N}M] \sim k_{NM}[bR]_{total}$, where k_{NM} is the decay constant of ^{N}M. To discuss more quantitatively, however, we have to distinguish the protons actually translocated from the protons released upon the formation of the long-lived photoproducts.

We estimated the quantum efficiency of the light-initiated conversion $N_{560} \rightsquigarrow bR_{570}$ by analyzing subsecond absorption kinetics of an alkaline suspension of pm fragments in the presence of background light. The model shown in Figure 1 predicts that, when the intensity of continuous light is suddenly decreased (e.g., when actinic light is turned off), the concentration of N_{560} decreases to a new level with a rate constant that is a function of the intensity of background light: $[N](t) = [N](\infty) + \{[N](0) - [N](\infty)\}\exp(-kt)$ and $k = k_N + (\phi_{N\rightarrow bR}\epsilon_N + \phi_{bR\rightarrow N}\epsilon_{bR})I_b$, where k_N is the thermal relaxation rate of N_{560}, ϕ the quantum efficiency of the corresponding light-initiated reaction, ϵ the molar absorption coefficients at the wavelength of background light, and I_b the intensity of background light. The concentration change of N_{560} after actinic light was turned off was experimentally deduced from the absorption recovery (the slow component) at 580 nm, where the difference spectrum between N_{560} and bR_{570} exhibits a maximal amplitude. In 3 M KCl, at pH 9.8 and 25 °C, the rate constant of absorption recovery was found to increase from 0.63 to 0.73 s^{-1} as the intensity of background light (570 ± 6 nm) was increased from 0 to 0.17 mW/cm². From these rate constants, we calculated the quantum efficiency $\phi_{N\rightarrow bR}$ to be larger than 0.5. In this calculation, we corrected for the inner filter effect of background light (OD_{570} = 0.18) using $\phi_{bR\rightarrow N}$ = 0.3–0.6, ϵ_{bR} (570 nm) = 63 000 cm^{-1} M^{-1}, and ϵ_N(570 nm) = 50 000 cm^{-1} M^{-1} (Kouyama et al., 1988b). Thus, the light-initiated reaction $N_{560} \rightsquigarrow bR_{570}$ seems to play a significant role.

Two-Photon Cycle at Physiological pH. Even when the external pH was not very high, the two-photon cycle could be the dominant reaction in the presence of a light-induced pH gradient. That is, absorption changes due to the accumulation of N_{560} and ^{N}M were induced when the vesicle suspension at or below neutral pH was exposed to high light intensities for a long period (Figure 6). At the beginning of the illumination, however, the light-induced difference absorption spectrum observed was not different much from that observed in pm fragments, in which the one-photon cycle was dominant.

It has been shown theoretically and experimentally that illumination of a bR-containing vesicle generates a membrane potential which is then replaced gradually by a pH gradient (Westerhoff et al., 1979; Michel & Oesterhelt, 1980). Figure 6 suggests that the light-induced membrane potential, at least in our preparation of the envelope vesicle, has a rather small effect on the late bR photoreactions. Instead, these steps were found to be dependent strongly on the internal pH; i.e., the thermal relaxations of N_{560} and ^{N}M were inhibited whenever the internal pH became high (pH >8). This is consistent with the scheme that both the light reaction $N_{560} \rightsquigarrow {}^{N}M \rightarrow bR_{570}$ and the thermal relaxation $N_{560} \rightarrow bR_{570}$ are accompanied by proton uptake at the cytoplasmic surface (Kouyama et al., 1988b).

When the envelope vesicle suspension at neutral pH was irradiated by strong light for a long period, the absorption recovery at 410 nm after the illumination took place very slowly; the slowest component had a time constant longer than 1 s at room temperature. (Multiplicity in the observed time constants is probably due to variation in the magnitude of light-induced pH gradient from one vesicle to another.) If we assume that the decay rate of ^{N}M is a simple function of the internal pH, the internal pH, at least in some fraction of the envelope vesicles, must increase from neutral pH to pH 11 or higher. The pH change thus calculated is not unreasonably large, because we have previously shown that bR can generate pH gradients as large as 4 pH units (Kouyama et al., 1988a).

Intramembrane Proton Transfer and Proton Uptake. In the present study, we presented evidence that the thermal processes $N_{560} \rightarrow bR_{570}$ and $^{N}M \rightarrow bR_{570}$ are accompanied by proton uptake at the cytoplasmic membrane surface and are strongly pH dependent. The lifetime of M_{412} was not affected very much by the internal pH nor by the external pH of the envelope vesicle, and therefore the transition $M_{412} \rightarrow N_{560}$ is presumably an intramembrane proton transfer, i.e., from a proton donor within the membrane to the deprotonated Schiff base. We could not detect any large effect of membrane potential on the decay rate of M_{412}. That is, the absorption change at 410 nm observed just after the light was turned on was not different for the envelope vesicles and the pm sheets (Figure 6). This can be explained if the membrane permeability to other ions (e.g., K^+) was so high that light-induced membrane potential did not become large. Another possibility is that the distance of proton movement during the $M_{412} \rightarrow N_{560}$ transition is much smaller than the membrane thickness.

A large effect of membrane potential on the decay rate of M_{412} was previously reported (Quintanilha, 1980; Dubrovskii et al., 1983; Groma et al., 1984). But the contribution of ^{N}M to the absorption kinetics at 410 nm was not considered, and therefore reevaluation of their experimental data is required. It seems likely that the photoproduct which Dubrovskii et al. claimed to be M_{412} actually corresponds to ^{N}M, as judged from the response time of their apparatus (>10 ms). Groma et al. (1984) reported that the slow decay component in the 410-nm absorption transient was enhanced when a membrane potential was generated by background light. In their experiments, 50 mM HEPES was added so that, according to them, no large pH gradient was generated in the light. Their data can be readily explained if the decay process of N_{560} (not M_{412}) is voltage dependent. This possibility was also suggested from

the light-induced difference spectrum observed just after the light was turned on (Figure 6).

Investigation of the decay rate of N_{560} in the presence of membrane potential seems to be important. In fact, besides a ~1-ms component, a slower component (10–20 ms) was found in flash-induced electric signals of pm (Holz et al., 1988; Rayfield, 1985; Keszthelyi & Ormos, 1980). Drachev et al. (1984) previously claimed that M kinetics, proton uptake kinetics, and electric signals were all coupled in a kinetic sense and suggested that the proton uptake phase accounted for about 80% of the overall potential generating during the one cycle. Importantly, their experiments were carried out at neutral pH and at low ionic strength, where the lifetime of N_{560} may be comparable to or shorter than that of M_{412}. Later, these authors (Drachev et al., 1986) reported that the proton uptake phase was coupled to the decay of N_{560}, which they called P. Accordingly, their results do not exclude the possibility that a large fraction of the potential is generated during the transition $N_{560} \rightarrow bR_{570}$.

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