Effect of a Light-Induced pH Gradient on Purple-to-Blue and Purple-to-Red Transitions of Bacteriorhodopsin†

Akemi Nasuda-Kouyama,‡ Kazuya Fukuda,‡ Takayoshi Iio,§ and Tsutomu Kouyama*,‡

The Institute of Physical and Chemical Research, Hirosawa 2-1, Wako, Saitama 351-01, Japan, and Department of Physics, Faculty of Science, University of Nagoya, Chikusa, Nagoya 464-01, Japan

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ABSTRACT: Bacteriorhodopsin-containing vesicles that were able to alkalize the extravesicular medium by >1.5 pH units under illumination, i.e., inside-out vesicles, were reconstituted by reverse-phase evaporation with *Halobacterium halobium* polar lipids or exogenous phospholipids. Acid titration of a dark-adapted sample was accompanied by a color change from purple to blue (pK_a = 2.5–4.5 in 0.15 M K_2SO_4), and alkali titration resulted in the formation of a red species absorbing maximally at 480 nm (pK_a = 7 to >9), the pK_a values and the extents of these color changes being dependent on the nature of lipid. When a vesicle suspension at neutral or weakly acidic pH was irradiated by continuous light so that a large pH gradient was generated across the membrane, either a purple-to-blue or a purple-to-red transition took place. The light-induced purple-to-red transition was significant in an unbuffered vesicle suspension and correlated with the pH change in the extravesicular medium. The result suggests that the purple-to-red transition is driven from the extravesicular side, i.e., from the C-terminal membrane surface. In the presence of buffer molecules outside, the dominant color change induced in the light was the purple-to-blue transition, which seemed to be due to a large decrease in the intravesicular pH. But an apparently inconsistent result was obtained when the extravesicular medium was acidified by a HCl pulse, which was accompanied by a rapid color change to blue. We arrived at the following explanation: The two bR isomers, one containing *all-trans*-retinal and the other 13-*cis*-retinal, respond differently to pH changes in the extravesicular and the intravesicular medium. In this relation, full light adaptation was not achieved when the light-induced purple-to-blue transition was significant; i.e., only the 13-cis isomer is likely to respond to a pH change at the N-terminal membrane surface.

In the living cells of *Halobacterium halobium*, bacteriorhodopsin acts as a light-energy-converting proton pump that actively translocates protons across the cell membrane, and the resulting electrochemical proton gradient is utilized for ATP synthesis and other cellular processes [for reviews, see Stoeckenius et al. (1979), Khorana (1988), and Kouyama et al. (1988a)]. Bacteriorhodopsin (bR)[1] consists of seven helical rods oriented perpendicularly to the membrane, the C-terminus and the N-terminus facing the cytoplasmic side and the outside, respectively. One molecule of retinal is bound to a lysine residue in the protein via a protonated Schiff base linkage. When a bR isomer containing *all-trans*-retinal (bR_{570}) absorbs light, it undergoes a cyclic chemical reaction that drives proton translocation from the cytoplasmic side to the outside.

In previous studies, we prepared *Halobacterium halobium* envelope vesicles that under illumination generated a pH gradient as large as 4 pH units across the membrane (Kouyama et al., 1987, 1988a) and investigated the feedback effect of a light-induced pH gradient on the photocycle of bR_{570} (Kouyama & Nasuda-Kouyama, 1989). It was found that the turnover of the photocycle became very slow as the intravesicular pH increased in the light, causing an apparent decrease in the proton pumping activity. This slowdown was explained by the inhibition of the proton uptake at the cytoplasmic surface. However, we failed to detect any significant effect of the pH gradient itself, which has been widely believed to regulate the reaction kinetics of membrane-located proton-translocating systems (Mitchell, 1976). The original purpose of the present work was to develop an experimental system in which possible effects of the pH gradient itself could be easily distinguished from the effect of the pH change at the cytoplasmic membrane surface. For this purpose, an effort has been made to reconstitute inside-out bR vesicles that can alkalize the extravesicular medium by >1.5 pH units under illumination. Spectroscopic studies of such inside-out vesicles provided an unexpected result, however. It turned out that a large light-induced pH gradient transformed the neutral purple form of bR to an acidic blue form or to an alkaline red form.

This paper describes the effect of a light-induced pH gradient on the photodynamic equilibrium between several different ground states of bR. Spectroscopic properties of bR have been extensively studied with purple membrane, a two-dimensional crystal of bR. At physiological pH, dark-adapted purple membrane contains a 2:1 mixture of 13-*cis*- and *all-trans*-retinal isomers (Scherrer et al., 1989) and absorbs maximally at 560 nm. When the equilibrium shifts toward the all-trans isomer in the light (light adaptation), the absorption maximum shifts to 570 nm. At low light intensity,

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* Author to whom correspondence should be addressed.
‡ The Institute of Physical and Chemical Research.
§ University of Nagoya.

[1] Abbreviations: bR, bacteriorhodopsin; HhPL, *H. halobium* polar lipids; EPC, egg phosphatidylcholine; SBPC, soybean phosphatidylcholine; MES, 2-(*N*-morpholino)ethanesulfonic acid monohydrate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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the trans photocycle is approximated by the following scheme: $bR_{570} \rightsquigarrow K_{590} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{570}$ (the subscripts indicate the absorption maxima) (Kouyama et al., 1988b; Lozier et al., 1975). In the far-blue-shifted intermediate M_{412}, the retinal Schiff base is deprotonated. At acidic pH (pH ~2), the absorption maximum occurs around 605 nm, and the color is blue (Moore et al., 1978; Fischer & Oesterhelt, 1979; Mowery et al., 1979). The purple-to-blue transition can be induced by other methods, for instance, by deionization of purple membrane (Kimura et al., 1984) or by addition of a lipophilic anion (Padros et al., 1984). These behaviors are explained entirely by a decrease in the membrane surface pH (Szundi & Stoeckenius, 1987, 1989). The blue form contains a mixture of 13-cis and all-trans (40:60) isomers, and the isomer ratio varies only slightly upon light adaptation (Mowery et al., 1979). In its trans photoreaction, analogues of the K_{590} and L_{550} intermediates appear, but the formation of M_{412} is blocked (Mowery et al., 1979; Kobayashi et al., 1983; Chronister et al., 1986). The appearance of the blue form was usually explained by the protonation of a counterion near the Schiff base (Fischer & Oesterhelt, 1979), but a different explanation was recently given by Szundi and Stoeckenius (1987). Conformational changes in the protein, which may result in charge rearrangement near the Schiff base, are suggested to accompany the purple-to-blue transition (Kimura et al., 1984).

In the alkaline pH region, an aqueous suspension of purple membrane scarcely changes its color, unless the pH is increased so high (pH >13) that the deprotonation of the Schiff base is accompanied by the formation of a 460-nm-absorbing species (Druckmann et al., 1982). When purple membrane is suspended in 60% dimethyl sulfoxide, however, a red species absorbing maximally at 480 nm is formed at or above neutral pH (Pande et al., 1989b; Oesterhelt et al., 1973). The treatment of purple membrane with anesthetics also produces a 480-nm-absorbing species at neutral pH (Nishimura et al., 1985; Lin et al., 1989). This red form (bR_{480}) was previously believed to have a deprotonated Schiff base and confused with the 460-nm-absorbing species appearing at an extremely high pH. But excitation of bR_{480} generates an M-type photoproduct with an absorption maximum at 385 nm (Baribeau & Boucher, 1985; Henry et al., 1988). A recent Raman study (Pande et al., 1989a,b) has shown that bR_{480} has a protonated Schiff base. Baribeau and Boucher (1987) have previously shown that lipid-free detergent-free bR is red even at acidic pH; pK_a = 2.5. The red form was also found in vesicles reconstituted with soybean phosphatidylcholine (pK_a = 7.5; Lozier et al., 1976; Hwang & Stoeckenius, 1977). That only the trans isomer can form the 480-nm species is shown with native purple membrane (Pande et al., 1989b; Henry et al., 1988) or with bR mutants (Mogi et al., 1989).

For better understanding of the purple-to-blue or purple-to-red transition of bR, it is obviously important to determine from which side of the membrane these transitions are driven. Although there have been attempts to answer this question, the results reported are conflicting (Bakker-Grunwald & Hess, 1981; Lind et al., 1981; Druckmann et al., 1985). In this study, we modulated the extravesicular and/or intravesicular pH values of inside-out vesicles by irradiating them in the absence and presence of buffer molecules outside and monitored absorption changes in the presence of a light-induced pH gradient. The result suggested that the purple-to-red transition was regulated primarily by the extravesicular pH value. But the purple-to-blue transition took place in a more complicated manner. The mechanism of these color changes, as well as the effect of a light-induced pH gradient on the bR photoreactions including the light/dark adaptation, will be discussed.

Materials and Methods

Cells of *Halobacterium halobium* strain JW3 were grown on a complex medium consisting of a basal salt solution supplemented with amino acids (Engelman & Zaccai, 1980). Purple membrane fragments were isolated from washed cells according to the established procedure (Oesterhelt & Stoeckenius, 1974) or purchased from Dainippon Ink & Chemicals Co., Ltd. The cell envelope vesicles were prepared as described earlier (Kouyama & Nasuda-Kouyama, 1989). Polar lipids of *H. halobium* (HhPL) were prepared from the envelope vesicles following Lind et al. (1981). Soybean phosphatidylcholine (SBPC) was purified from asolectin according to the procedure of Kagawa and Racker (1971). Egg phosphatidylcholine (EPC), soybean asolectin, valinomycin, and CCCP were purchased from Sigma Co. Ltd.

Bacteriorhodopsin-containing vesicles were reconstituted by the reverse-phase evaporation method (Rigaud et al., 1983) with slight modifications. A mixture of 2.5 mg of purple membrane fragments in 0.5 mL of salt solution and 25 mg of lipids in 1.5 mL of diethyl ether (pH ~6) was sonicated at 0 °C in a bath-type sonicator (Shimadzu SUS-200, Kyoto, Japan) for 30–60 s; then, the solvent was evaporated under reduced pressure (~350 mmHg) at room temperature for 20–60 min; when the dispersion became viscous, 1.5 mL of salt solution was added, and the evaporation was continued for >30 min to remove traces of diethyl ether. After the removal of debris by centrifugation at 3000 rpm for 10 min (only for HhPL vesicles), reconstituted vesicles were kept at room temperature (or dialyzed against a desired solvent) for 1 day; this incubation was required for the complete recovery of the visible absorption band, which had been partly lost during the sonication. (We refer to bR-containing vesicles reconstituted with HhPL, EPC, and SBPC as HhPL, EPC, and SBPC vesicles, respectively.) In the above preparation procedures, the salt composition of the solution was found to be an important factor affecting the vesicle quality, which was estimated from the magnitude of the light-induced pH change. That is, a high salt concentration (3 M KCl) was required for the reconstitution of HhPL vesicles, whereas EPC vesicles were well reconstituted in 0.12 M K_2SO_4. SBPC vesicles were also reconstituted in 0.12 M K_2SO_4, but a better condition remains to be found. From the extent of extrusion of reconstituted vesicles through Nuclepore membrane filters, the vesicle size was estimated to be ~0.4 μm for EPC and SBPC vesicles and <0.2 μm for HhPL vesicles. (When HhPL vesicles were extruded through a membrane filter with a pore size of 0.2 μm, the turbidity decreased significantly, but the amplitude of the visible absorption band scarcely changed, suggesting that large vesicles were composed of almost pure lipids.)

Measurements of the light-induced pH change were performed with a glass electrode (type GK2321; Radiometer, Copenhagen, Denmark) connected to a Horiba M8s pH meter, as described earlier (Kouyama et al., 1987).

Absorption spectra in the presence of intense actinic light 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. Actinic light from an Xe lamp was passed through a heat-absorbing water filter, reflected from a cold mirror (400–700 nm), passed through optical filters (>540 nm), and then focused into a sample cell. Measuring light, provided from a Shimadzu UV350a spectrophotometer, was passed through the sample cell from the

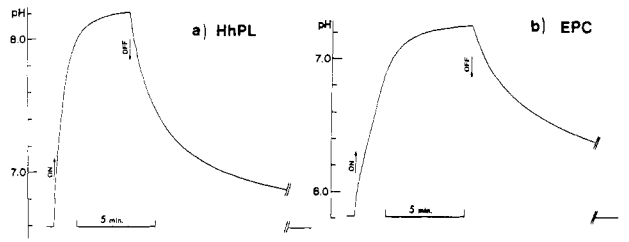

FIGURE 1: Light-induced pH changes in suspensions of reconstituted vesicles at 25 °C. (a) HhPL vesicles containing 19 nmol of bR in 0.6 mL of 0.6 M K_2SO_4 (+40 mM KCl). (b) EPC vesicles containing 16 nmol of bR in 0.6 mL of 0.6 M K_2SO_4 (+1 μM valinomycin). The vesicle suspension in a cuvette (1-cm diameter) with a magnetic stirrer was irradiated by yellow light (500–660 nm) at 40 mW/cm^2, and pH changes were monitored under Ar gas flow with a glass pH electrode.

opposite direction to the actinic light and reflected onto a photomultiplier tube (Hamamatsu R374, Hamamatsu Japan). Millisecond absorption kinetics measurements were performed with a constant-power Xe flash lamp (Nissin Electronic Co., Ltd., Tokyo, Japan) providing light pulses of 10-μs width. Light pulses, above 540 nm and at a frequency of 0.25 Hz, irradiated the sample cell at a right angle to the measuring light. The amplified photosignal was fed to a digital memory scope (Iwatsu DS6121, Tokyo, Japan) operated in a pretrigger mode. Absorption spectra in which the light-scattering effect was minimized were recorded with a sample cell holder that was placed close to the photomultiplier tube. Acid-induced absorption changes in the millisecond time scale were measured with a Union-Giken RA-401 stopped-flow spectrophotometer; the instrumental dead time was 4 ms.

RESULTS

(1) Light-Induced pH Changes in Reconstituted Vesicles. Figure 1 shows examples of light-induced alkalization of the extravesicular medium of HhPL and EPC vesicles. The largest pH change so far investigated (1.7 pH units) was induced when EPC vesicles were irradiated in the presence of valinomycin, a K^+-specific ionophore. But valinomycin was not always required for the observation of large light-induced pH changes. In fact, the extent of light-induced proton uptake into HhPL vesicles (Figure 1a) was little affected by valinomycin. Instead, the presence of a high concentration of sulfate (0.6 M K_2SO_4) was always required. At a low concentration (0.12 M K_2SO_4), light-induced pH changes were at largest 1.3 pH units. In the absence of sulfate, light-induced pH changes were usually small (<0.5 pH unit in 3 M KCl; <1.1 pH units in ~1 M KCl). It should be mentioned that vesicle preparations were not always successful. Some samples showed no large pH changes even in 0.6 M K_2SO_4 and, in the worst case, exhibited a biphasic pH response upon irradiation. In the following experiments, the vesicle preparations capable of alkalizing the extravesicular pH by >1.5 pH units under illumination were used, with a few exceptions. Also, we avoided using a high concentration of valinomycin in optical measurements, because the binding of valinomycin to bR is known to affect the photoreactions of bR (Avi-Dor et al., 1979; Hasselbacher & Dewey, 1986).

The apparent proton pumping activity of bR in reconstituted vesicles was highest at weakly acidic pH and decreased in the alkaline pH region. In Figure 2, the initial rate of light-induced proton uptake into HhPL and EPC vesicles is plotted against the extravesicular pH. The examples shown were obtained when vesicles were suspended in 3 M KCl. When 3 M KCl was replaced by 0.12–0.6 M K_2SO_4, the decrease in the apparent activity at high pH became more remarkable; especially

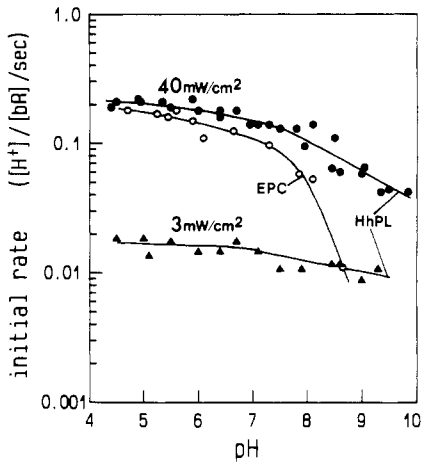

FIGURE 2: Initial rate of light-induced proton uptake into EPC vesicles (○) and HhPL vesicles (●, ▲). The vesicle suspensions containing 11–15 nmol of bR in 0.6 mL of 3 M KCl, 0.5 mM HEPES, and 0.5 mM MES (+10 μM valinomycin for EPC vesicles) at 30 °C were irradiated by yellow light (500–600 nm) at 40 (○, ●) or 3 mW/cm^2 (▲). The quantities shown were calculated from the initial part (a few seconds) of the light-induced pH change, in which the pH change was apparently linear with time.

in vesicles reconstituted with exogenous lipids, light-induced pH changes were almost undetectable above pH 8. (The reason for a more significant pH dependence in K_2SO_4 than in 3 M KCl will become clear later.) Thus, large light-induced pH changes as shown in Figure 1 were observed only when the extravesicular pH of unbuffered vesicle suspensions was adjusted at a weakly acidic value before illumination, i.e., at pH 5–6 for EPC vesicles and at pH 6–7 for HhPL vesicles. When the pH was further lowered, the magnitude of the light-induced pH change became smaller. This was explained partly by a higher buffering capacity of the vesicle outside and partly by a decrease in the extent of light-induced proton uptake at such a low pH that some fraction of the bR molecules were transformed into the blue form (see below). In 3 M KCl (+0.5 mM HEPES and MES), for instance, the extent of light-induced proton uptake into HhPL vesicles was highest at pH 5–8 (~3 H^+ per bR). It should be noted that the extent of light-induced proton uptake was small ($[H^+]/[bR] < 6$) even when a pH change larger than 1.5 pH units was induced.

The small extent of proton uptake is related to a low buffering capacity of the vesicle inside. Figure 3 shows the pH titration curves of an unbuffered suspension of HhPL vesicles in 1.2 M KCl. In the figure, the open circles represent the buffering capacity of the vesicle outside (*minus* the buffering capacity of the medium), and the solid triangles represent the buffering capacity of the vesicle inside. These quantities were calculated from the amplitudes of a fast pH decrease after a HCl pulse and of a slow subsequent pH increase. At pH 4–6, the buffering capacity of the vesicle inside was much smaller than that of the vesicle outside. This is consistent with the structural model of bR (Engelman et al., 1980), in which much fewer carboxyl groups distribute on the N-terminal membrane surface than on the other membrane surface. [A sharp increase in the buffering capacity below pH 3.5 is explained by the phosphate groups of phospholipids; the major lipid component of HhPL, phosphatidylglycerol phosphate, was recently shown to have the terminal phosphate O-methylated (Fredrickson et al., 1989; Tsujimoto et al., 1989).] From the titration curves shown in Figure 3 and the number of protons taken up in the light, one can estimate the magnitude of the light-induced pH decrease in the vesicle inside. In the example shown in Figure 1a, ~4.5 protons per

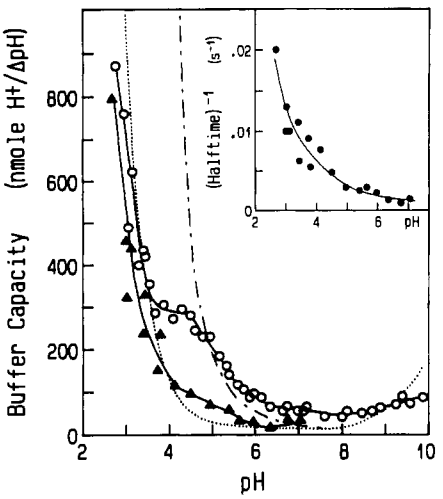

FIGURE 3: pH titration of HhPL vesicles containing 32 nmol of bR: (○) buffering capacity of the vesicle outside *minus* buffering capacity of the medium (dotted line; 0.6 mL, 1.2 M KCl); (▲) buffering capacity of the vesicle inside. When a HCl pulse was applied to a dark-adapted suspension under Ar gas flow, the extravesicular pH decreased quickly and then increased slowly; from the amplitudes of the fast pH decrease and the slow subsequent pH increase, ΔpH_{fast} and ΔpH_{slow}, the buffering capacities of the vesicle outside and inside, B_{out} and B_{in}, were calculated with the following approximation: $B_{out}(pH_{01}) = [H^+]/\Delta pH_{fast}$ and $B_{in}(pH_{0\infty})/B_{out}(pH_{1\infty}) = \Delta pH_{slow}/\Delta pH_{fast}$, where $pH_{01} = (pH_0 + pH_1)/2$, $pH_{0\infty} = (pH_0 + pH_\infty)/2$, and $pH_{1\infty} = (pH_1 + pH_\infty)/2$. pH_0 and pH_∞ represent the initial and final pH values, respectively, and pH_1 is the extravesicular pH observed just after addition of HCl. The buffering capacity of the vesicle inside was not determined at high pH (due to a nonnegligible pH drift). This quantity was also difficult to determine in 0.6 M K_2SO_4, because of a strong buffering action of the medium at acidic pH (the broken line; 0.6 mL, 0.6 M K_2SO_4). Inset: The inverse of the halftime of the slow subsequent pH increase after a HCl pulse (at 25 °C) is plotted against the medium pH (the average of initial and final pH values); each HCl pulse caused a pH change of ~0.35 (±1.5) pH unit. In 1.2 M KCl, the pK_a value of the purple-to-blue transition was 3.5.

bR were taken up, and the intravesicular pH was calculated to decrease to pH ~4.2. That is, the pH gradient generated across the membrane (~4 pH units) was close to the maximal value so far reported. When the extravesicular pH was kept below neutral pH (in 0.6 M K_2SO_4) so that ~6 protons per bR were taken up in the light, the intravesicular pH was calculated to decrease below pH 4. In these calculations, we assumed that the vesicle population was homogeneous in the orientation of bR and in the permeability to proton. Also, the buffering capacity of the vesicle inside was assumed to be unchanged when 1.2 M KCl was replaced by 0.6 M K_2SO_4; this replacement scarcely affected the pK_a value of the purple-to-blue transition (pK_a ~ 3.5). [In 0.6 M K_2SO_4, the titration curves as shown in Figure 3 were difficult to obtain, because the buffering capacity of the medium was very high at acidic pH (the broken line in Figure 3). Because the volume of the vesicle inside was much smaller (≪0.05) than that of the vesicle outside, the buffering action of sulfate ions existing in the vesicle interior would be negligible unless the intravesicular pH decreased far below pH 4.]

(*2*) *Absorption Spectra of Reconstituted Vesicles in the Dark.* A dark-adapted suspension of HhPL vesicles (pH 6) absorbed maximally at 562 nm. When the vesicle suspension was made acidic (pH <3) in the dark, the absorption maximum shifted to ~590 nm. The acid-induced difference spectra obtained (Figure 4a) are very similar in profile to those reported for native purple membrane (Moore et al., 1978; Mowery et al., 1979; Kimura et al., 1984). When HhPL vesicles were alkalized (pH >9), on the other hand, an absorption decrease at 570 nm was accompanied by an absorption

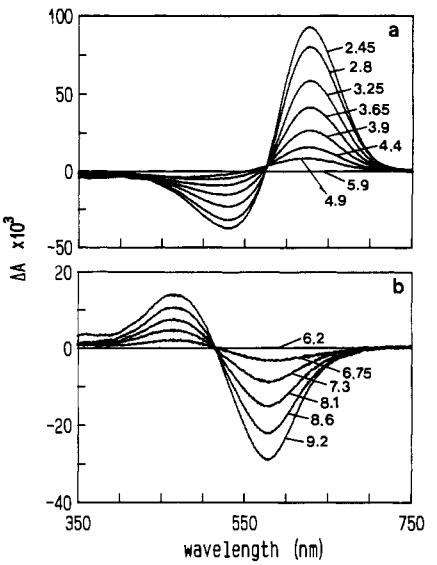

FIGURE 4: Difference spectra induced by acidification (upper panel) and alkalization (lower panel) of a dark-adapted suspension of HhPL reconstituted vesicles. Absorbance at 560 nm was 0.165 at pH 6. Solvent: 1 M KCl.

increase at 460 nm (Figure 4b). A similar absorption change has been shown to occur upon treatment of native purple membrane with dimethyl sulfoxide or anesthetics and explained by the formation of a 480-nm-absorbing speces (bR_{480}) (Pande et al., 1989a; Nishimura et al., 1985).

In vesicles reconstituted with exogenous lipids, i.e., in EPC and SBPC vesicles, the neutral form of bR absorbed maximally at ~550 nm in the dark-adapted state. (Suspensions of EPC and SBPC vesicles were turbid, and the accurate position of the absorption maximum was difficult to determine.) Reflecting a ~10-nm blue-shift of the absorption spectrum as compared with that of HhPL vesicles, the acid- and alkali-induced difference spectra observed for EPC and SBPC vesicles are slightly blue-shifted as compared with those shown in Figure 4. More importantly, the apparent pK value and the extent of purple-to-blue or purple-to-red transition were dependent strongly on the nature of the lipid as well as on the salt composition of the medium. The results obtained are summarized in Figure 5, in which data obtained for the cell envelope vesicles are also presented. In vesicles containing negatively charged lipids, i.e., in HhPL and SBPC vesicles, the pK_a value of the purple-to-blue transition decreased with increasing concentration of salt. This dependence was not seen in EPC vesicles. With respect to the purple-to-red transition, a relatively low pK_a value (pK_a ~ 7) was found in vesicles reconstituted with exogenous lipids, but no general rule was found about the effect of ionic strength. To our surprise, the envelope vesicles also exhibited a purple-to-red transition in 0.6 M K_2SO_4. This transition was inhibited in 3 M KCl (the closed circles in figure 5b). In general, a high concentration of chloride ion stabilized the purple form in the alkaline pH region.

(*3*) *Color Changes Induced in HhPL Vesicles under Continuous Illumination.* Flash-induced absorption changes in HhPL vesicles were not very different from those found in native purple membrane. In 3 M KCl at pH 6 and 20 °C, for instance, the halftime of the absorption recovery at 410 nm was 5.0 ms in HhPL vesicles, which was only slightly longer than the value (3.5 ms) found in native purple membrane. A little longer halftime in HhPL vesicles was due to the presence of a slowly decaying component (τ ~43 ms) with a relative amplitude of ~20%. (This slow component was

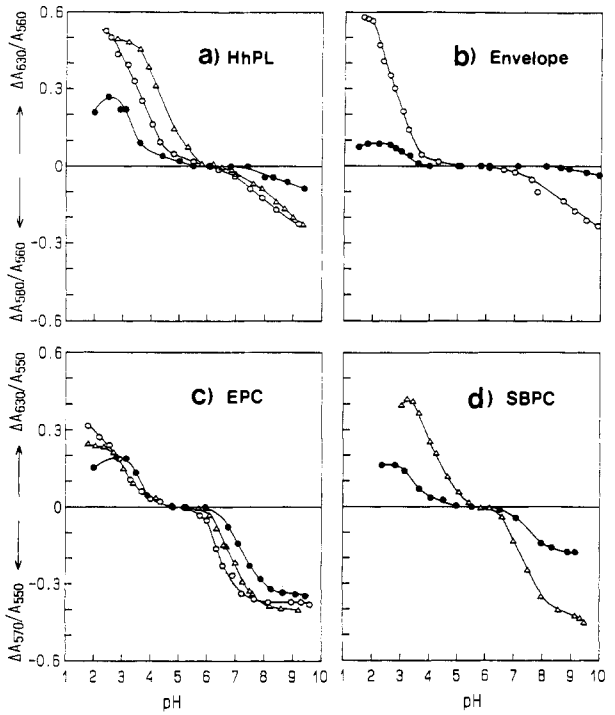

FIGURE 5: Absorption increase at 630 nm induced by acidification (the upper part of each panel) and absorption decrease at 580 or 570 nm induced by alkalization (the lower part) of dark-adapted suspensions of reconstituted vesicles and the cell envelope vesicles (at 25–30 °C). Data obtained in 3 M KCl (●), 0.6 M K_2SO_4 (○), and 0.12 M K_2SO_4 (△) are presented. The data of EPC and SBPC vesicles were obtained in the presence of 1–3 μM valinomycin.

dominant in vesicles reconstituted with exogenous lipids.) When HhPL vesicles were exposed to continuous light, however, the absorption changes induced were completely different from those observed in purple membrane fragments. Figure 6 shows examples of absorption changes when HhPL vesicles in 0.6 M K_2SO_4 were irradiated by orange light (540–700 nm) at 30 mW/cm^2. The results obtained were strongly dependent on whether or not the extravesicular medium contained buffer molecules. The left panels in Figure 6 show data for an unbuffered suspension, in which a large pH change (pH 7.0 → 8.5) was induced by yellow light at 40 mW/cm^2 (Figure 1a). The right panels show data for a buffered suspension (5 mM HEPES buffer at pH 7.0), in which the light-induced pH change in the extravesicular medium was restricted within 0.05 pH unit. From the difference spectra shown in figure 6, it appears that the following reactions took place in the light: (1) the photocycle of bR_{570} (the closed squares in Figure 6b,e); (2) the purple-to-red transition (the broken line in Figure figure 6b); (3) the purple-to-blue transition (the broken line in Figure 6e). The dark/light adaptation also took place (traces e in Figure 6c,f; also see Figure 8). These reactions are described below in more detail.

We first fix attention on the data shown in Figure 6a. When a partly dark-adapted suspension of HhPL vesicles was exposed to orange light, the absorbance at 590 nm quickly decreased and, after a short constant phase, again decreased with a time constant of ~20 s. The fast absorption change was attributable to the photocycle of bR_{570}, and the slowly decreasing component was explained by the purple-to-red transition (see below). The second constant phase was not seen at 550 nm and was replaced by an absorption-increasing phase at 630 nm. The wavelength dependence of its amplitude suggested that the second phase resulted from the light adaptation. (In HhPL vesicles, the difference spectrum associated with the light adaptation was shown to have a large positive peak at

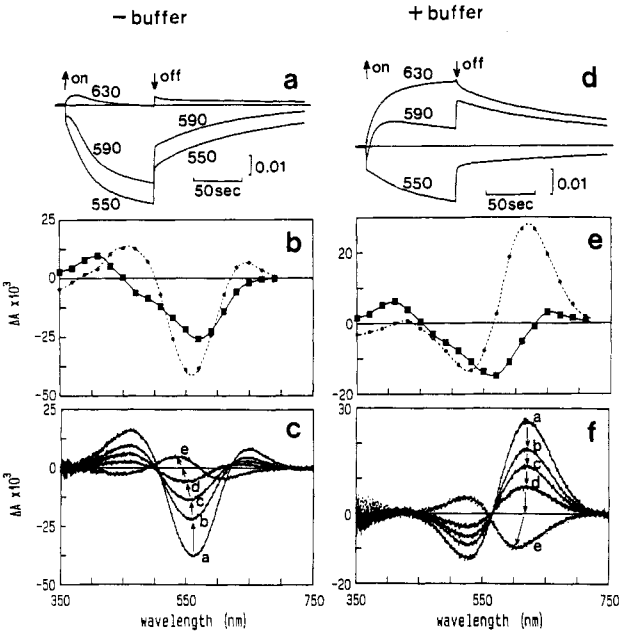

FIGURE 6: Light-induced absorption changes in HhPL-reconstituted vesicles in the absence (left) and presence (right) of buffering molecules outside. The sample used in Figure 1a was transferred into a cuvette of 2-mm optical length (OD_{562} = 0.4), and the inside of the cuvette was filled with nitrogen gas. The sample was irradiated by orange light (540–700 nm) at an intensity of 30 mW/cm^2. Left: The same solvent condition as used in Figure 1a, except that the pH was adjusted at pH 7.0 in the dark-adapted state. Right: 5 mM HEPES buffer (pH 7.0) added. Top: The vesicle suspension was irradiated for 90 s every 600 s, and light-induced absorption changes were recorded at various wavelengths. Middle: The amplitudes of the fast absorption change seen just after the 90-s illumination (■) and the subsequent absorption change taking place during 510 s (○) are plotted as a function of wavelength. Bottom: The dark-adapted suspension was irradiated for 5 min, and then, absorption spectra were recorded with various delays; i.e., the wavelength scan from 750 to 350 nm at a speed of 800 nm/min was started 0, 1, 2, and 4 min (traces a–d), 8 min (base line), and 16 min (trace e) after the light was turned off; the absorption spectrum recorded 8–8.5 min after the illumination was used as a base line to calculate the difference spectra shown.

590 nm and a small negative peak at 500 nm, as in native purple membrane; see Figure 8.) When the actinic light was turned off, a quick absorption change due to the photocycle was followed by a slow absorption change. In Figure 6b, the amplitude of the slow component is plotted as a function of wavelength (the broken line). A negative peak is seen at 560 nm, and positive peaks are seen at 460 and 650 nm. This difference spectrum is similar in profile to the alkali-induced difference spectrum (Figure 4b), except for the long-wavelength region. The small peak at 650 nm seemed to be due to the purple-to-blue transition, which was morre significant in the buffered suspension (see below). The time course of absorption recovery after 5-min illumination is shown in Figure 6c, in which the absorption spectrum recorded 8 min after the light was turned off is used as a base line. The negative band at 560 nm diminished with a half time of ~3 min, and this absorption change was approximately coincident with the pH recovery after the illumination (Figure 1a). These observations suggested that a pH gradient across the membrane and/or a pH increase in the extravesicular medium was responsible for the purple-to-red transition induced in the light.

When the extravesicular medium contained 5 mM HEPES buffer, the dominant color change induced in the light was the purple-to-blue transition (Figure 6d–f). When the light was turned on, the absorbance at 590 nm quickly decreased, subsequently increased with a time constant of a few seconds, and then decreased slowly (Figure 6d). The fast absorption change

was attributed to the photocycle of bR_{570}, and the subsequent increase was explained partly by the light adaptation and partly by the purple-to-blue transition (see below). To explain the slowly decreasing component, however, we need to postulate another reaction. This component was most significant in the wavelength region (570–590 nm) where the absorption decrease due to the dark adaptation was largest and the absorption increase due to the purple-to-blue transition was relatively small. It will be shown later that full light adaptation does not take place when the purple-to-blue transition is significant. The most likely explanation of the slow component is that a large decrease in the intravesicular pH inhibited the light adaptation and/or accelerated the dark adaptation very much. [The light reaction of the blue form leading to a 9-cis-containing species with λ_{max} at 480 nm (Maeda et al., 1980; Fischer et al., 1981; Chang et al., 1987) might occur, but its contribution was undetectable under the present experimental conditions; see Figure 8.] Although the light-induced absorption changes were complicated by unusual dark/light adaptation in the presence of a pH gradient, the data in Figure 6d–f suggested that the purple-to-blue transition was induced in the light. The broken line in Figure 6e represents the slow component of the absorption change observed when the actinic lilght was turned off. This difference spectrum has a positive peak at 620 nm and a negative peak at 470 nm, and its global profile is similar to the acid-induced difference spectra (Figure 4a). Figure 6f shows the absorption recovery after 5-min illumination. In difference spectrum a, a positive peak is seen at ~615 nm. This peak drifted to a shorter wavelength with time, and in trace e, the profile became rather similar to, but still more red-shifted than, the difference spectrum associated with the dark adaptation. As seen in the pH recovery (Figure 1a), the back-reaction of the purple-to-blue transition was fast just after the light was turned off, but it lasted >10 min. (Also, the dark adaptation seemed to be faster in the presence of a larger pH gradient, contributing difference spectra a–d which are slightly blue-shifted as compared with the acid-induced difference spectrum.) The halftime of ~4 min for restoring purple color is roughly the same as that for the pH recovery, suggesting that the pH decrease in the intravesicular medium is a major factor in the purple-to-blue transition.

The photocycle of bR_{570} was not affected much by a light-induced pH gradient, as long as the extravesicular pH was fixed. The closed squares in Figure 6e represent the amplitude (inverted) of the fast absorption change observed when the actinic light was turned off. Positive peaks occur at 410 and 640 nm. These positive peaks are presumably due to the formation of M_{412} and O_{640} (Lozier et al., 1975). Because a similar absorption change was induced when a light-adapted suspension of purple membrane was irradiated (Kouyama & Nasuda-Kouyama, 1989), the last parts of the photocycle of bR_{570} were suggested to be unchanged even when the pH gradient became so large that some fraction of bR molecules became blue.

When the extravesicular pH was allowed to freely increase in the light, however, the last parts of the trans photocycle changed significantly. In the difference spectrum shown by the closed squares in Figure 6b, the positive peak at long wavelength was missing; i.e., the O_{640} intermediate became difficult to detect as the extravesicular pH increased in the light. A large negative peak at ~570 nm in the difference spectrum is indicative of the accumulation of a larger amount of N_{560} at a higher extravesicular pH, because the difference spectrum between N_{560} and bR_{570} is characterized by a negative peak at 580 nm (Kouyama et al., 1988b).

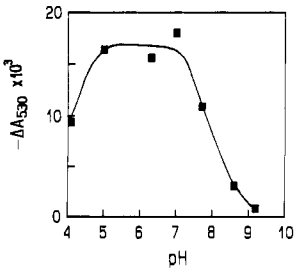

FIGURE 7: Extent of light-induced purple-to-blue transition in HhPL vesicles versus the external pH. Difference spectra as shown in the bottom of Figure 6 were obtained in the presence of 5 mM HEPES and 5 mM MES at various pH values; the spectra recorded 0–0.5 and 16–16.5 min after illumination were compared, and the absorption difference at 530 nm between the two spectra was calculated.

(*4*) *Effect of the Medium pH on Light-Induced Purple-to-Blue and Purple-to-Red Transitions in HhPL Vesicles.* The light-induced purple-to-red transition was most significant when the medium pH was initially adjusted at 6.5–7.5 (in 0.6 M K_2SO_4). In the alkaline pH region investigated, the extent of the light-induced purple-to-red transition was approximately proportional to the light-induced pH increase in the extravesicular medium, which was largest (>1 pH unit) at the initial pH of between 5.5 and 7.5 and decreased at higher pH (~0.5 pH unit at the initial pH of 8.5). When the initial pH was low (pH <5.5), the purple-to-red transition was not induced in the light; instead, the purple-to-blue transition was induced. The absence of a light-induced purple-to-red transition at low pH was readily explained by the fact that, in the dark-adapted state, the absorption change associated with the purple-to-red transition was seen only above pH 6.5 (Figure 5a). These observations suggest that the purple-to-red transition is entirely governed by the extravesicular pH. Because the light-induced purple-to-red transition was not observed in the presence of 5 mM HEPES, neither the decrease in the intravesicular pH nor the pH gradient itself is responsible for the purple-to-red transition. (It will be shown later that the membrane potential is not effective at inducing this transition.)

As compared with the purple-to-red transition, the purple-to-blue transition took place in a much more complex manner. In Figure 7, the amplitude of the slow absorption recovery at 530 nm after the 5-min illumination is plotted as a function of the extravesicular pH, which was kept at an almost constant value during the illumination. The absorption change at 530 nm can be used as a measure of the extent of the light-induced purple-to-blue transition, because the dark/light adaptation was accompanied by a negligible absorption change at 530 nm (see Figure 8b). The small extent of light-induced purple-to-blue transition at pH 4 is presumably related to the fact that a considerable fraction (20–30%) of bR molecules had already been transformed to the acidic form before the illumination (Figure 5). The extent of light-induced purple-to-blue transition also decreased when the extravesicular pH was kept at a high pH (pH >8). This pH dependence apparently suggests that, with a high extravesicular pH given, the internal pH cannot decrease below a critical value that would be required for the purple-to-blue transition. However, it should be noted that the extent of light-induced purple-to-blue transition was much smaller than expected from the absorption change induced by the complete acid titration. This is the case even when the extravesicular medium was acidic (Figure 7). In this relation, we observed that, when the extravesicular medium was fast acidified by a HCl pulse, a purple-to-blue shift took place in the millisecond time scale (this will be shown in Figure 10). This result alone may suggest that the purple-to-blue transition is driven from the

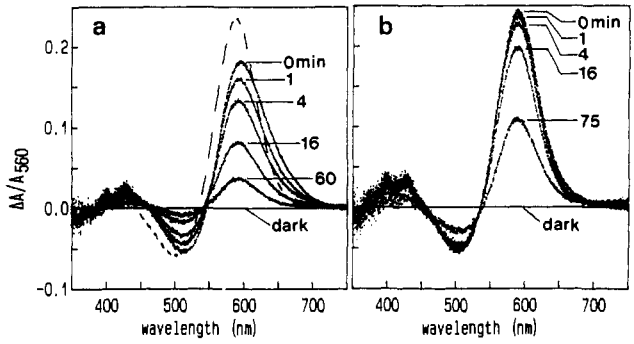

FIGURE 8: Light/dark adaptation and light-induced purple-to-blue transition in HhPL vesicles: (a) in 0.6 M K_2SO_4, 40 mM KCl, and 10 mM HEPES at pH 6; (b) in 3 M KCl and 10 mM HEPES at pH 8. Dark-adapted suspensions ($OD_{560} \sim 0.6$) at 25 °C were irradiated by orange light (30 mW/cm², 540–700 nm) for 15 (a) or 5 min (b), and absorption spectra were recorded with various delays after the light was turned off; the wavelength was scanned from 750 to 350 nm at a speed of 800 nm/min. The difference spectra shown were calculated with the absorption spectrum of the dark-adapted state as a base line. The broken line in the right panel represents the difference spectrum induced when purple membrane was light adapted in 0.6 M K_2SO_4.

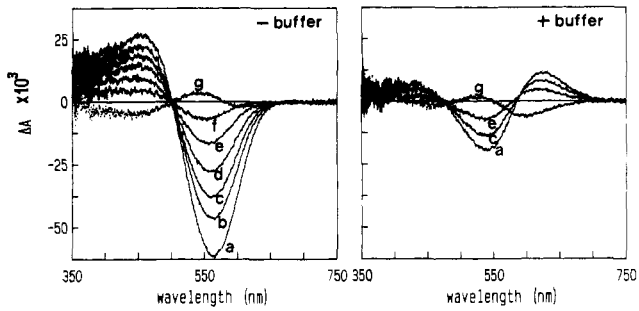

FIGURE 9: Light-induced absorption changes in EPC-reconstituted vesicles. Left: The sample used in Figure 1b (pH 5.8 in the dark-adapted state) was transferred into a cuvette with an optical length of 2 mm ($OD_{550} = 0.28$). Right: 10 mM phosphate buffer (pH 6.0) was added. The vesicle suspension was irradiated by orange light (540–700 nm) at 30 mW/cm² for 10 min, and then, the absorption spectra were recorded with various delays; i.e., the wavelength scan was started 0, 1, 2, 4, 8, and 16 min (traces a–f), 32 min (base line), and 60 min (trace g), after the light was turned off. The absorption spectrum recorded at 32–32.5 min was used as a base line for the calculation of the difference spectra shown.

extravesicular membrane surface. These apparently inconsistent observations will be discussed later.

(*5*) *Light/Dark Adaptation and Purple-to-Blue Transition in HhPL Vesicles.* To solve the above complex problem, one needs to understand the light/dark adaptation of bR in the presence of a large pH gradient. It was found that full light-adaptation did not take place whenever the light-induced purple-to-blue transition was significant. Figure 8 shows absorption changes observed when a dark-adapted suspension of HhPL vesicles in 0.6 M K_2SO_4 or in 3 M KCl was irradiated. In 3 M KCl at pH 8 (10 mM HEPES), no significant purple-to-blue transition was induced in the light. [This is presumably related to the low pK_a value of this transition in 3 M KCl (Figure 5a).] In such a case, the light/dark adaptation took place normally; i.e., the difference absorption spectrum associated with the light adaptation was very similar in profile and in amplitude to that observed for native purple membrane (the broken line in Figure 8a). In 0.6 M K_2SO_4 at pH 6, on the other hand, significant light-induced purple-to-blue transition took place, and its contribution to the absorption changes observed was so large that the absorption change due to light adaptation was concealed considerably (Figure 8a). At the wavelength (570–575 nm) where the contribution of the purple-to-blue transition was negligible, the absorbance increase was much smaller than expected from full light-adaptation. After the light was turned off, the absorbance at this wavelength decreased faster in 0.6 M K_2SO_4 than in 3 M KCl. As suggested in Figure 6d, this result also suggests that the dark adaptation became faster when the purple-to-blue transition was more significant. (In purple membrane, the time constant of dark adaptation was 70 ± 10 min at pH 6–8 and 25 °C, independent of whether 3 M KCl or 0.6 M K_2SO_4 was used.)

There was a strong correlation between the two phenomena, light-induced purple-to-blue transition and the light/dark adaptation. At pH 8 in 0.6 M K_2SO_4, light-induced purple-to-blue transition was still observable; then, the light adaptation did not take place fully, but its extent was higher than observed at pH 6. In 3 M KCl, the purple-to-blue transition could be induced in the light, provided that the outside medium was acidic (pH 5); in this case, full light adaptation did not take place. This correlation suggests that only the 13-*cis*-bR isomer undergoes the purple-to-blue transition in the light. This possibility will be discussed later in more detail.

(*6*) *Light-Induced Color Changes in Vesicles Reconstituted with Exogenous Lipids.* The light-induced purple-to-red transition was seen more clearly in EPC vesicles. One example is presented in the left panel of Figure 9, which was obtained when an unbuffered suspension of EPC vesicles in 0.6 M K_2SO_4 (+1 μM valinomycin) was irradiated. In this example, absorption recovery after illumination took place very slowly ($t_{1/2} \sim 4$ min), reflecting the slow pH recovery (Figure 1b). In EPC vesicles, the alkali-induced purple-to-red transition was completed in a narrow pH region (between pH 5.4 and pH 8.2; Figure 5). Due to this sharp pH dependence, a large absorption change was induced when the extravesicular pH was varied around neutral pH. For instance, the absorption change observed during incubation in the dark for 32 min after 10-min illumination ($\Delta A_{570}/A_{550} = 0.21$; Figure 9, left) accounted for about 60% of the absorption change induced by the complete alkali titration. For a quantitative comparison of the light-induced absorption changes and pH changes (Figures 1b and 9, left), the following factors should be taken into account: (1) the actinic light used in the absorption measurement was less intense than that used in the pH measurement; (2) a small but finite amount of right-side-out vesicles coexisted (see below). Thus, the amplitude of the light-induced absorption change observed is not very different from the value that would be deduced on the assumption that the purple-to-red transition is governed by the extravesicular pH.

When EPC vesicles in 0.12 M K_2SO_4 were irradiated in the absence of valinomycin, the pH changes induced were very small (<0.1 pH unit), and the purple-to-red transition was undetectable. The small pH change is presumably due to a low permeability of this lipid membrane to potassium ion, because the light-induced pH change was enhanced very much upon addition of valinomycin. In the absence of valinomycin, a large membrane potential was expected to be generated in the light. Thus, the absorption change observed suggested that the membrane potential was not effective in inducing the purple-to-red transition.

Unlike HhPL vesicles, EPC vesicles scarcely exhibited the light-induced purple-to-blue transition. The result obtained in the presence of 10 mM phosphate (pH 6) is presented in the right panel of Figure 9. Besides a small amplitude of the purple-to-blue transition, the purple-to-red transition also contributed to the difference spectra a–c in the figure. This

component suggested coexistence of right-side-out vesicles, because their intravesicular pH was expected to increase in the light. In the example shown, right-side-out vesicles may account for ~10% of the total vesicles.

In SBPC vesicles, the light-induced purple-to-blue transition was found to take place to a similar extent as in HhPL vesicles (not shown). This light-induced color change was most significant at a weakly acidic pH (pH 5–7 in 0.12 M K_2SO_4) and enhanced by valinomycin but diminished in the presence of CCCP and valinomycin. This result suggests that the decrease in the intravesicular pH is responsible for the light-induced purple-to-blue transition. (We did not investigate the light-induced purple-to-red transition in SBPC-vesicles, because optical measurements were allowed only for a diluted suspension, in which no large change in the extravesicular pH was induced in the light.)

Discussion

(1) Effect of a Light-Induced pH Gradient on the Equilibrium between Different Ground States of bR. Using inside-out reconstituted vesicles, we have shown here that the late part of the photocycle of bR_{570} is affected by the pH at the C-terminal membrane surface. For instance, the O_{640} intermediate became difficult to detect as the extravesicular pH increased in the light (Figure 6b). This is not a direct effect of the pH gradient itself, because the trans photocycle did not change very much when the intravesicular pH decreased at a fixed value of the extravesicular pH (Figure 6e). The same conclusion has been previously derived from the spectroscopic studies of the cell envelope vesicles (Kouyama & Nasuda-Kouyama, 1989). In reconstituted vesicles, however, we found new effects of a light-induced pH gradient: (1) When the extravesicular pH was allowed to freely increase in the light, the equilibrium between the neutral purple form and the alkaline red form shifted toward the red form. (2) When the extravesicular pH was kept at a constant value, the decrease in the intravesicular pH was accompanied by the formation of the acidic blue form. (3) The dark adaptation was accelerated by a pH gradient and/or by a decrease in the intravesicular pH.

The above three phenomena may be coupled with one another. First, the purple-to-red transition seems to affect the equilibrium between the two bR isomers, because the 13-cis isomer has been reported to be resistant to the purple-to-red transition (Pande et al., 1989b; Henry et al., 1988). Second, the apparent acceleration of dark adaptation by a light-induced pH gradient may result from a light-induced purple-to-blue transition, because the thermal conversion between the *all-trans*- and the 13-*cis*-bR isomers has been suggested to be very rapid in the blue form (Mowery et al., 1979). Another possibility is that only the 13-cis isomer undergoes the purple-to-blue transition as the intravesicular pH decreases; in this case, rapid dark adaptation is a prerequisite for the light-induced purple-to-blue transition (see below). Third, the purple-to-blue transition and the purple-to-red transition could be induced simultaneously in some circumstance (Figure 6c), and therefore, these two transitions cannot be regarded as completely independent phenomena. If the intravesciular medium is made very acidic while the extravesicular pH is kept at a very high value, an equilibrium may be attained between the red form with *all-trans*-retinal and the blue form with 13-*cis*-retinal. Below we discuss the observed phenomena separately, but the possible couplings between them should be kept in mind.

(2) Purple-to-Red Transition. The present result shows that the equilibrium between the neutral purple form and the alkaline red form is determined, as a first approximation, by the extravesicular pH of inside-out vesicles; i.e., the purple-to-red transition is driven from the C-terminal membrane surface. Previous studies of the reactivity of the red form with hydroxylamine have indicated that the red form has the retinal Schiff base exposed to the medium (Oesterhelt et al., 1973). It is likely that the red form assumes such a protein conformation in which an aqueous channel penetrates from the C-terminal membrane surface to the Schiff base. But the 13-cis isomer is resistant to the purple-to-red transition (Pande et al., 1989b; Henry et al., 1988; Mogi et al., 1989). That is, 13-*cis*-retinal is not accommodated comfortably in the red form. Although the crystalline structure of purple membrane is retained in the red form (Pande et al., 1989b), the protein conformation near the retinal binding site seems to be largely altered. This does not necessarily mean that the conformation change is very localized. In fact, the equilibrium between the purple form and the red form was strongly dependent on the lipid environment; it was also affected by the salt composition of the medium (Figure 5). The charge density at the cytoplasmic membrane surface may have a significant role.

(3) Purple-to-Blue Transition. The mechanism of the purple-to-blue transition is not simple. We observed the following two phenomena, which are apparently inconsistent with each other: (1) the purple-to-blue transition took place as the intravesicular pH decreased in the light; (2) fast acidification of the extravesicular pH by a HCl pulse was accompanied by a rapid color change from purple to blue (Figure 10a), as has been previously observed by Lind et al. (1981). The former result appears to suggest that the purple-to-blue transition is driven from the N-terminal membrane surface, whereas the latter result seems to suggest that this color change is driven from the other membrane surface. Previous discussions about this problem are also conflicting. Druckmann et al. (1985) carried out stopped-flow experiments with inside-out vesicles with and without a buffer enclosed in the vesicle interior. From the observation of a buffer-induced delay in the formation of the blue form, they concluded that the retinal Schiff base and its counterion were exposed to the N-terminal membrane surface. The opposite answer was suggested by the previous observation that positively charged polymers [(diethylaminoethyl)dextran] inhibited the purple-to-blue transition in inside-out vesicles (Bakker-Grunwald & Hess, 1981), though the authors emphasized that the result was not conclusive. Several researchers insisted that deionized blue membrane restored its purple color upon binding of divalent cations to the C-terminal membrane surface (Chang et al., 1986; Duñach et al., 1987; Roux et al., 1988), though the effect of the cation binding was explained differently by Szundi and Stoeckenius (1989).

To explain these apparently contradictory data, we consider the possibility that the two bR isomers, one containing *all-trans*-retinal and the other containing 13-*cis*-retinal, respond differently to the pH change in the extravesicular or intravesicular pH. That is, the following reactions are proposed to take place under illumination or in the dark. (1) When the intravesicular pH of the inside-out vesicles decreases in the light, the dark adaptation is accelerated, and the 13-cis isomer undergoes a purple-to-blue transition; the trans isomer does not change its color as long as the extravesicular pH is fixed; under continuous illumination, an equilibrium may be attained between the blue form with 13-*cis*-retinal and the purple form with *all-trans*-retinal. (Rapid dark adaptation at low intravesicular pH will be discussed later.) (2) When the extravesicular medium is acidified by a HCl pulse in the dark, the

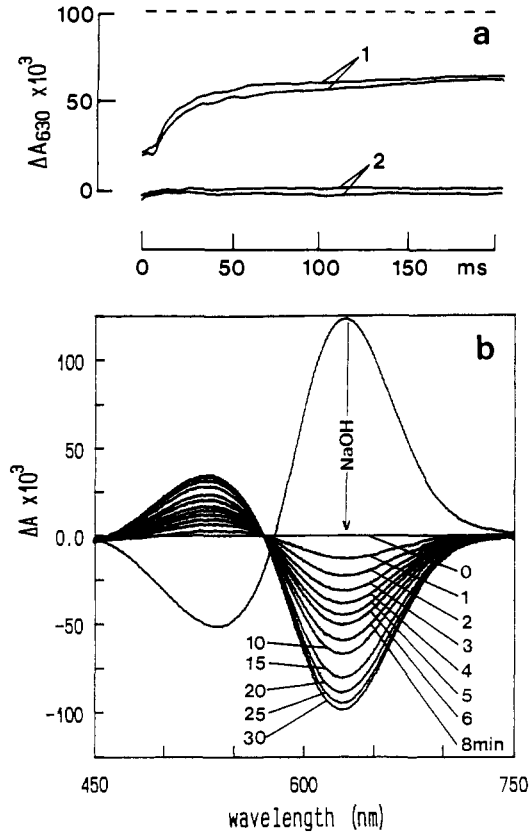

FIGURE 10: (a) Acid-induced absorption kinetics at 630 nm of HhPL vesicles in 0.15 M KCl at 20 °C. Traces 1 were obtained when the suspension at pH 5.9 was mixed with an acidic KCl solution so that the final pH became 4.25. Traces 2 were obtained when the suspension mixed with the same solvent at pH 5.9. The broken line represents the absorbance that would be expected 10 min after the pH decrease. In 0.15 M KCl, the pK_a value for the purple-to-blue transition was 4.9. (b) Absorption changes after addition of NaOH to an acidic suspension of HhPL vesicles in 1.2 M KCl at 15 °C. The initial pH was 3.2, and NaOH was added so that the final pH became 4.4; the halftime of a slow pH decrease following a fast pH increase was ~8 min; the pK_a value for the purple-to-blue transition was 3.5. The absorption spectrum recorded just after addition of NaOH (the dead time = 15 s) was used as a base line for calculation of the difference spectra shown. The wavelength was scanned from 750 to 450 nm at a speed of 800 nm/min.

trans isomer changes its color rapidly; a subsequent slow proton leak into the vesicle inside causes the 13-cis isomer to undergo a purple-to-blue transition. (3) Upon addition of NaOH to an acidic suspension, the transition to the purple form is rapid for the trans isomer but slow for the 13-cis isomer. The proposal is consistent with the above statement that in the red form only the *trans*-retinal chromophore is accessible from the C-terminal membrane surface. Experimental data supporting the proposal are presented below.

First, the light-induced purple-to-blue transition was not always significant (e.g., in EPC vesicles). Even in HhPL and SBPC vesicles, a rather small fraction of bR molecules were converted to the blue form as a large pH gradient was generated in the light; i.e., the absorption increase at 630 nm due to the light-induced purple-to-blue transition was maximally 20% of that induced by the complete acid titration (Figures 7 and 8). More importantly, full light adaptation was not achieved whenever the light-induced purple-to-blue transition was significant; ~30% of the total bR molecules may remain in the 13-cis state (Figure 8a). These results are consistent with the proposal that only the 13-*cis*-bR isomer undergoes the purple-to-blue transition as the intravesicular pH of inside-out vesicles decreases in the light. One might argue that the vesicle population is not homogeneous in the orientation of bR; e.g., a small fraction of bR molecules with right-side-out orientation in an otherwise perfect inside-out vesicle may be responsible for the purple-to-blue transition induced in the light. But the light-induced purple-to-blue transition was significant only when high-quality vesicles were used. Thus, it does not seem likely that all the data shown are explained by inhomogeneity in the vesicle population, though its minor contribution cannot be ruled out.

Careful analyses of absorption kinetics induced by a HCl or NaOH pulse also support our proposal. Figure 10a shows an example of acid-induced absorption kinetics at 630 nm of a suspension of HhPL vesicles. There was a fast component ($t_{1/2}$ ~ 20 ms), which accounted for ~50% of the total absorption increase. But there was also a slow absorption change that lasted ~1 min. In the example shown in Figure 10b, NaOH was added to an acidic suspension of HhPL vesicles so that a fast pH increase in the outside medium was followed by a slow pH decrease with a halftime of ~8 min. Under this experimental condition, ~50% of the total absorption change at 630 nm was explained by a fast component (<30 s), and the rest was explained by a slow component with a halftime of ~10 min. This slow absorption change is likely to reflect the slow pH increase in the vesicle inside. These results suggest that there are two bR species, one responding to the intravesicular pH and the other to the extravesicular pH. It should be noted that the difference spectrum associated with the slow component was slightly more blue-shifted than that of the fast component. This is readily explained, because the absorption spectrum of the neutral purple form is more blue-shifted in the 13-cis state than in the trans state. It is known that the kinetics of the purple-to-blue transition or its reverse reaction are not described by a single time constant; upon addition of divalent cation to the blue form, for instance, there are three kinetically distinct steps with time constants ranging from a few milliseconds to several seconds (Druckmann et al., 1979; Fischer & Oesterhelt, 1979; Kimura et al., 1984; Zubov et al., 1986). A better description of these complex kinetics may be achieved on the assumption that the two bR isomers respond differently to pH changes.

Our proposal is consistent with the experimental data of Druckmann et al. (1985), because their inside-out vesicles are likely to contain the 13-cis isomer. Druckmann et al. (1985) rationalized their conclusion from the fact that excitation of the *trans*-bR isomer resulted in a rapid release of a proton from the N-terminal membrane surface. But all the early intermediates in the trans photocycle contain 13-*cis*-retinal (Smith et al., 1986). A difference is expected in the proton accessibility to the Schiff base and its counterion(s) for the ground state and its photoproducts. Of course, we are now discussing the 13-*cis*-retinal with a syn C15=N bond in the dark-adapted state, which is not identical with the 13-*cis*-retinal with an anti C15=N bond in the early intermediates of the trans photocycle. To discuss the protein conformations of the photocycle intermediates, a more through investigation is required.

Because we did not consider possible effects of the pH gradient rigorously, our proposal may require modifications. It may be more reasonable to assume that the equilibrium between the purple form and the blue form is determined by a weighted average of the pH values at the two membrane surfaces, the weighting factor being different for the two bR isomers. This modification will not be necessary, however, if one accepts the assumption that the protonation of a counterion(s) near the Schiff base is responsible for the purple-to-blue transition. Then, it is unlikely that the Schiff base in

a fixed protein conformation is able to detect the two pH values, because it would mean that fast proton movement is allowed from the Schiff base to both sides of the membrane: i.e., it would mean that any bR-containing vesicles are very permeable to proton. To avoid this implausible conclusion, one may have to abandon the above assumption, as suggested by Szundi and Stoeckenius (1989). An alternative answer is: The Schiff base detects the two pH values by using two different protein conformations that are thermally interconvertible. In the latter case, the interconversion between them should be slow so as not to allow fast passive proton movement through the protein. One possible candidate for such an interconversion process is the dark adaptation of bR. In this relation, we observed that the proton permeability of HhPL vesicles became higher at such a low pH that the purple-to-blue transition took place (Figure 3). Because the pH dependence of the proton permeability was more significant in vesicles containing bR molecules than in pure lipid vesicles, the protein is likely to mediate rapid proton leakage at low pH. This result is consistent with our proposal, because the interconversion between the two bR isomers becomes faster at lower pH; its halftime is ~20 min at neutral pH, and it becomes as short as ~30 s at acidic pH (in 0.2 M KCl at 30 °C) (Ohno et al., 1977).

(*4*) *Dark/Light Adaptation of bR in Reconstituted Vesicles.* In HhPL vesicles, the dark adaptation became very fast as the intravesicular pH decreased in the light at a fixed value of the extravesicular pH. This observation may not be surprising, because the dark adaptation of purple membrane has been shown to become fast at acidic pH (Ohno et al., 1977). Lind et al. (1981) previously observed that the extent of light adaptation of bR incorporated into HhPL vesicles (inside-out vesicles) was small in the absence of ionophores and that it became larger when CCCP and valinomycin were added. The fast dark adaptation at a low intravesicular pH can explain, at least partly, the small extent of light adaptation in inside-out vesicles. However, little information is available about the couplings among the photoreactions of different ground states, and presently, we cannot exclude another reaction that contributes to reduce the extent of light adaptation. For instance, it is possible that a branching reaction from the trans cycle to the 13-cis cycle becomes significant in the presence of a large pH gradient. Casadio and Stoeckenius (1980) previously observed dark adaptation by light in bR monomers. This light-induced dark adaptation may be related to the light-induced purple-to-blue transition. But we do not know the exact behavior of bR monomers that may exist in our vesicles. Also, we do not know what will occur if the 13-*cis*-bR isomer is excited in the presence of a large pH gradient. Hellingwerf et al. (1978) previously investigated flash-induced absorption changes at 660 nm in inside-out vesicles with and without background illumination. Besides the fast absorption change due to the decay of O_{640}, they observed a very slowly decaying component ($\tau > 0.1$ s) in the presence of background light. Although they attributed this slow component to a change in the turbidity, it might be due to photoreactions of ground states other than bR_{570}. In any case, a more comprehensive investigation of the feedback effect of a light-induced proton gradient on the bR photoreactions is required.

(*5*) *Optimal Solvent Conditions for a Large Light-Induced pH Change.* In our vesicle preparations, only ~6 protons per bR were maximally taken up under the steady illumination, but a light-induced pH change in the extravesicular medium could be as large as 1.7 pH units. Such a large pH change was not reported previously, except for the cell envelope vesicles. In most previous studies, the quality of reconstituted vesicles was estimated from the extent of light-induced proton uptake (or proton release in the case of right-side-out vesicles), $[H^+]/[bR]$. Obviously, each bR molecule can translocate more protons when a smaller number of bR molecules are incorporated into a larger lipid vesicle. At a low protein concentration, however, the magnitude of the light-induced pH change can be easily reduced by a relatively higher permeability to proton and by a relatively larger buffering capacity of the extravesicular medium, especially in the presence of dissolved CO_2.

A nonuniform orientation of the proteins in reconstituted vesicles also contributes to reduce the magnitude of the light-induced pH change. Up to date, many methods have been developed for functional reconstitution of bR into lipid vesicles [for reviews, see Racker (1979) and Dencher (1988)]. A method that provides a uniform orientation of the proteins is of course desirable. In this regard, the detergent-mediated method developed by Huang et al. (1980) is most successful, though the preparation procedure is not simple. With respect to the reverse-phase evaporation method, Seigneuret and Rigaud (1985) reported that both inside-out and right-side-out orientations of bR were present in relative amounts of 80% and 20%, respectively. Nonetheless, this method has the following advantage: The two orientations are highly segregated so that bR molecules have an inside-out orientation in one vesicle and a right-side-out orientation in another vesicle; the resulting two subclasses of vesicle are separable (Seigneuret & Rigaud, 1988). In this work, the separation procedure, which resulted in a considerable dilution of samples, was omitted; instead, we selected such samples that exhibited a large light-induced pH change. In the preparations used, right-side-out vesicles may account for ~10% of the total vesicles. The contribution of such a vesicle fraction to the light-induced pH change can be minimized, however, by selective inactivation of the bR molecules in right-side-out vesicles. This selective inactivation seemed to be substantially achieved under such solvent conditions that the purple-to-red transition took place significantly, because the red form translocated protons less actively than the purple form did (Figures 2 and 5) and because the buffering capacity of reconstituted vesicles was lowest at weakly alkaline pH; remember that the intravesicular pH of right-side-out vesicles increases in the light. We observed a larger light-induced pH change in 0.12–0.6 M K_2SO_4 than in 3 M KCl. This may be related to the fact that the purple-to-red transition took place more significantly in 0.12–0.6 M K_2SO_4 than in 3 M KCl (Figure 5). But a strong buffering action of sulfate ions at low pH could be a major factor that enhanced the extent of proton uptake into inside-out vesicles; we observed that, when sulfate was added to a suspension of HhPL vesicles in KCl, the magnitude of the light-induced pH change increased gradually (several hours at 25 °C). We may also have to consider contributions of the "acid-purple" form that appears at very low pH in the presence of Cl^- (Fischer & Oesterhelt, 1979), because this form was recently reported to work as a light-driven chloride pump (Dêr et al., 1989).

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