Effects of Chloride and pH on the Chromophore and Photochemical Cycling of Halorhodopsin†

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ABSTRACT: We have recently demonstrated that halorhodopsin, a retinal protein in *Halobacterium halobium* membranes, is a light-driven chloride pump [Schobert, B., & Lanyi, J. K. (1982) *J. Biol. Chem. 257*, 10306–10313]. In this study, we examine the effects of chloride and protons on the chromophores of halorhodopsin and bacteriorhodopsin, with the expectation that these ions, substrates of the two light-driven pumps, respectively, will interact with the apoproteins sufficiently to perturb the retinal moiety. All experiments were with cell envelope vesicles at high salt concentrations, which preserved transport activity, i.e., either in 4 M NaCl or in 1.5 M Na_2SO_4 with added NaCl. We find that in contrast with the photocycle of bacteriorhodopsin, whose major kinetic component shows sharp pH dependence, the flash-induced absorbance changes in halorhodopsin are virtually independent of pH between 5 and 9. Chloride, on the other hand, which does not affect bacteriorhodopsin under these conditions, has profound effects on the photocycle of halorhodopsin: after addition of chloride, the flash yield for halorhodopsin is greatly enhanced, and the time for recovery from flash bleaching is increased about 4-fold. Halorhodopsin, which has an absorption band near 590 nm, exhibits complex pH and chloride-dependent spectral shifts not observable in bacteriorhodopsin. When vesicles containing halorhodopsin are suspended in Na_2SO_4 at alkaline pH, addition of chloride causes the appearance of an absorption band at 590 nm and the disappearance of a band at 410 nm. Hence, in the absence of chloride, a reversible shift from 590 to 410 nm must have taken place. A 410-nm species is produced also in the presence of chloride, but only by illumination at alkaline pH. This photoreaction is only partially reversible in the dark. We interpret these results in terms of a scheme in which halorhodopsin is in equilibrium with a chloride-bound form, which exhibits a different and more efficient photocycle. Both forms of halorhodopsin are in a pH-dependent equilibrium with deprotonated forms, in which the chromophore is unperturbed. We propose that in these forms the retinal Schiff's base proton is lost spontaneously when chloride is absent, but only upon illumination when chloride is present. Chloride therefore appears to stabilize the protonated Schiff's base at alkaline pH. The apparent affinity constant for chloride in producing all of the effects described here is the same as the K_m for chloride transport (about 40 mM), suggesting that this behavior of the chromophore is relevant to the transport function of the pigment.

Evidence has become available recently that halobacteria contain two other retinal proteins, in addition to bacteriorhodopsin: halorhodopsin and the so-called "slowly cycling rhodopsin", or sR. Halorhodopsin absorbs near 590 nm (Lanyi & Weber, 1980; Weber & Bogomolni, 1981) and undergoes a photocycle with a recovery time of about 10 ms, similar to that of bacteriorhodopsin (Weber & Bogomolni, 1981). "Slow rhodopsin" also absorbs near 590 nm but shows a much slower photocycle with a recovery time of about 1 s (Bogomolni & Spudich, 1982; Tsuda et al., 1982). The latter pigment does not seem to generate a membrane potential upon illumination and may be related to photosensory functions (Bogomolni & Spudich, 1982).

We have found (Schobert & Lanyi, 1982) that halorhodopsin in cell envelope vesicles behaves not as an outward-directed sodium pump (Lindley & MacDonald, 1979; MacDonald et al., 1979) but as an inward-directed chloride pump. It is not known whether the chloride translocation is coupled directly to the photochemical events in halorhodopsin or to the internal migration of another ion, such as protons. One might expect, however, that the presence of chloride at its binding site would influence, directly or indirectly, the halorhodopsin chromophore and its photochemical reactions. Such effects were postulated for protons in bacteriorhodopsin, a proton pump [for a review, see Stoeckenius et al. (1979)]. Although the absorption spectrum of this pigment is virtually insensitive to pH between 4 and 10.5, the photocycling rate and pathway are considerably affected by the proton concentration. At pH below 6, recovery of the pigment in purified purple membranes is increasingly through the intermediate designated as O_{640}, a species largely absent at higher pH (Lozier & Niederberger, 1977). The rate of rise for the M_{412} photointermediate increases with pH, while the rate of decay falls (Stoeckenius et al., 1979; Scherrer et al., 1981; Lam & Packer, 1982). The total flash-induced absorbance change was reported by Ort & Parson (1978) to be relatively pH independent, but the fast kinetic component of the decay, which dominates the kinetics at pH 6, falls off markedly at pH 9 and above. Renard & Delmelle (1981) found, however, that the accumulation of the intermediate M_{412} in purified purple membrane was pH insensitive, in apparent contrast to an earlier report that in whole *Halobacterium* cells the yield of M_{412} increases considerably with pH between 6 and 9 (Wagner & Hope, 1976). It is difficult to separate pH effects in bacteriorhodopsin due to the binding of transported protons from those which involve the ionization state of groups necessary to stabilize the structure of the protein. We expect that this kind of ambiguity in interpretation will not be a problem for halorhodopsin because chloride binding is inherently more specific than proton binding. Chloride-dependent red shifts of 20–40 nm have been observed in the Gecko visual pigment (Crescitelli, 1977) and in chicken cone iodopsin (Fager & Fager, 1979), but their functional significance in the physiology of these systems is not known.

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The chromophoric properties of halorhodopsin were described earlier (Lanyi & Weber, 1980; Ogurusu et al., 1981). Some of its properties are similar to those of bacteriorhodopsin, such as the capacity to be bleached by hydroxylamine in the light and reconstituted with added retinal, indicating that this pigment very likely also contains a retinal–lysine Schiff's base linkage. Other properties, such as the heat sensitivity of haloopsin (Matsuno-Yagi & Mukohata, 1977), an apparent lack of dark adaptation, and a reversible blue shift near pH 10 (Lanyi & Weber, 1980), distinguish halorhodopsin from bacteriorhodopsin, although not necessarily from slow rhodopsin.

It now appears that the photocycle of halorhodopsin includes three intermediates, absorbing at 632, 500, and 380 nm, arising in linear sequence after the absorption of a photon (Weber & Bogomolni, 1981). The overall cycle time is about 10 ms, similar to that of bacteriorhodopsin. In the absence of NaCl, the photocycle of halorhodopsin is drastically altered: the intermediates found absorb at 630 and 640 nm, and the overall cycle time is shortened to 2.5 ms (Bogomolni et al., 1981). Slow rhodopsin cycles through a different set of intermediates, absorbing at 680 and 373 nm, and its recovery rate is much slower, about 0.8 s (Bogomolni & Spudich, 1982).

The spectrophotometric study of the three different pigments in the halobacteria is facilitated by the availability of stable *Halobacterium halobium* strains which lack one or another of the retinal proteins. The presence or absence of various light-responsive pigments in these strains is characterized by flash spectroscopy under defined conditions (Weber & Bogomolni, 1982; Bogomolni & Spudich, 1982; Spudich & Spudich, 1982) and measurement of the kind of ionic gradients created during illumination of cells or cell envelope vesicles (Wagner et al., 1981; Lanyi & Oesterhelt, 1982; Schobert & Lanyi, 1982). If it is assumed that any functional retinal protein in the halobacterial membrane will exhibit photochemical activity, it seems unlikely from the available information that a fourth such protein is present in these mutants in anything but trace quantities. Likewise, the existence of a third light-dependent transport system seems unlikely.

The most valuable strains for this study have been those which lack bacteriorhodopsin. Since at this time no *H. halobium* strain is available which contains halorhodopsin but not slow rhodopsin, examination of the properties of halorhodopsin alone is difficult. However, differences in photocycling kinetics (Bogomolni & Spudich, 1982) and in the rate of bleaching with hydroxylamine (this study) have allowed us to compare the effects of chloride, pH, and other influences on the two pigments.

Materials and Methods

The *Halobacterium halobium* strains (Wagner et al. 1981) used were L-33 (bacterioopsin minus), JW-5 (retinal minus, formerly designated as ET 1001/25), and L-07 (bacterioopsin and retinal minus). Since the biosynthesis of haloopsin is tightly controlled by retinal (Spudich et al., 1983), membranes from the last two strains regenerated virtually no halorhodopsin after retinal was added. Our results here indicate that the amount of slow rhodopsin in reconstituted JW-5 membranes is negligible compared to bacteriorhodopsin. Thus, the strain L-33 could be used for membranes containing halorhodopsin and slow rhodopsin, the strain JW-5 for membranes containing bacteriorhodopsin, and the strain L-07 for membranes containing slow rhodopsin alone. The procedure for growth of the cells and preparation of cell envelope vesicles was described earlier (Lanyi & MacDonald, 1979). The experiments with vesicles were carried out either in 4 M NaCl or in 1.5 M Na_2SO_4. For the latter, the vesicles (originally in 4 M NaCl) were dialyzed in the dark at room temperature against 1.5 M Na_2SO_4, adjusted to pH 6.5. Sodium sulfate vesicles, kept at room temperature, were used within a day or two, whereas NaCl vesicles could be stored in the refrigerator for months without loss of activity. Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

The retinal pigments were bleached by intense illumination at 10 °C in the presence of 0.2 M NH_2OH in 4 M NaCl (Lanyi & Weber, 1980) at various pHs, as described under Results. The length of illumination depended on the membrane concentration: at 8 mg/mL protein, complete bleaching was accomplished only in 36–48 h. The excess hydroxylamine was removed by extensive washing (Lanyi & Weber, 1982). Whenever substitution of NaCl with Na_2SO_4 was desired in addition to bleaching, the sample was dialyzed against the sulfate instead of being washed. Removal of NH_2OH by this method was not complete, however. Reconstitution of the pigments was with *all-trans*-retinal, added in MeOH solution. Difference spectra were determined with a Gilford Model 2600 spectrophotometer. Scanning was typically with 1.8–2.4-nm bandwidth, and two readings were averaged every 0.5 nm.

Transport activity of halorhodopsin was determined by following the light-dependent generation of membrane potential, as described earlier (Schobert & Lanyi, 1982).

The photocycle of the retinal pigments was followed with a cross-beam flash photometer designed for highly turbid samples. It consisted of a 250-W quartz–halogen lamp (EJL) and a Schoeffel 0.25-m monochromator set at 570 nm (measuring beam) and a Photochemical Research Associates Model 610B pulsed light source outfitted with a long-pass 610-nm filter (actinic beam). The vesicle suspension was in a 10 × 4 mm fluorescence cuvette, which was placed with its short path parallel to the measuring beam and offset toward the flash source so that the measuring beam impinged on the edge of the sample. The detector, a 2-in. EMI 9558 photomultiplier protected with two 570-nm interference filters, was placed directly behind the cuvette. The signal was amplified with a Tektronix 3A9 differential amplifier and acquired in a Nicolet 1072 signal averager. Flash frequency was 4 Hz; the signal averager was triggered, after a suitable delay, by circuitry in the flash unit. Flash duration was about 0.01 ms; the signal acquisition was typically at 0.10 ms/point. Flash discharge was at 5 or 6 kV, which corresponded to flash energies well below saturation under the conditions used. Signal averaging was for 128–512 flashes. The turbidity of the membrane preparations at the concentrations used (10 mg/mL protein) was high, giving absorbance values of 2–2.5 at 570 nm. However, results with bacteriorhodopsin in envelope vesicles were not very different from those obtained with purified purple membranes, which scatter very little, suggesting that artifacts due to turbidity are not serious.

Results

Resolution of the Photocycles for the Three Halobacterial Retinal Pigments. Photochemical activity of bacteriorhodopsin, halorhodopsin, and slow rhodopsin was followed by determining bleaching and recovery at 570 nm following flashes of light above 610 nm. These wavelengths were suitable for observing the photoinduced changes in all three pigments. Figure 1A shows that JW-5 membranes, which contain predominantly bacteriorhodopsin after reconstitution with retinal, recover from flash bleaching with near-first-order kinetics of a 7.6-ms half-life [for a discussion of the bacteriorhodopsin photocycle, see Stoeckenius et al (1979)]. Deviation from first order, which in this trace accounts for about the last tenth of

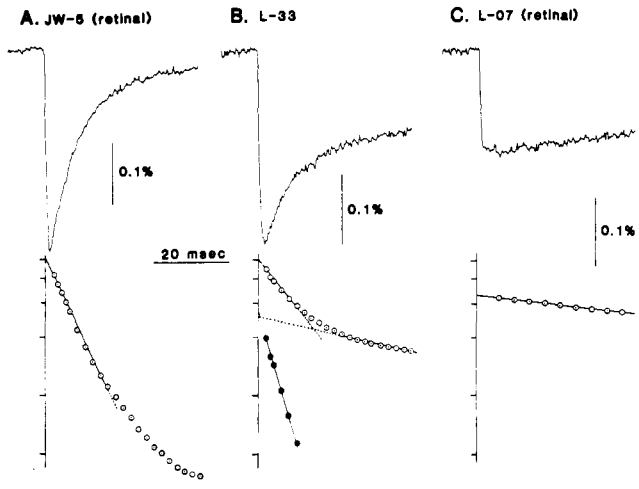

FIGURE 1: Flash bleaching and recovery traces for membranes prepared from three *H. halobium* strains. The cell envelope vesicles were suspended in 4 M NaCl, pH 6.5 (10 mg/mL protein). (A) JW-5 membranes, reconstituted with retinal (1.0 nmol/mg of protein, with essentially all retinal reacted with the bacterioopsin). Photoactive pigment is nearly exclusively bacteriorhodopsin. (B) L-33 membranes. Photoactive pigments are halorhodopsin and slow rhodopsin. (C) L-07 membranes, reconstituted with retinal (0.25 nmol/mg of protein). Photoactive pigment is slow rhodopsin. The kinetic analyses under the traces use the usual semilog plotting and curve peeling. In (B), the composite trace (O—O) is resolved into a rapid (●—●) and a slow (O--O) component. Measuring light, 570 nm; actinic light, above 610 nm; conditions for determining flash-induced absorbance changes as described under Materials and Methods.

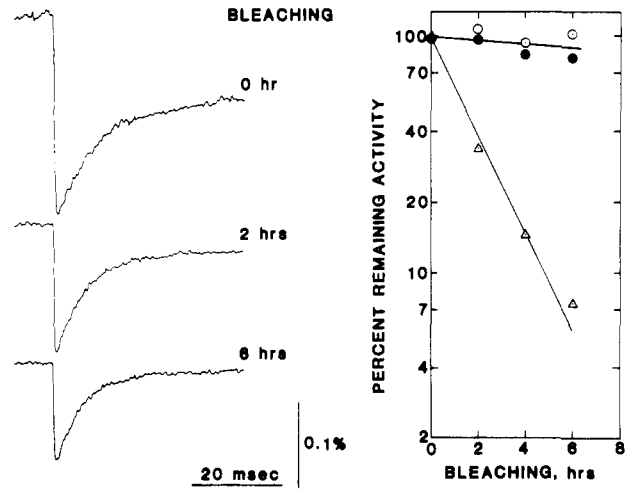

FIGURE 2: Differential hydroxylamine bleaching of halorhodopsin and slow rhodopsin L-33 vesicles (10 mg/mL) were subjected to bleaching for 0, 2, and 6 h at pH 7, as described under Materials and Methods, and flash-induced absorbance changes, as well as the light-dependent membrane potential (transport activity), were determined. On the left, the traces show preferential removal of slow rhodopsin during bleaching. On the right, the percent remaining photochemical activity (flash yield) of halorhodopsin (O), the transport activity of halorhodopsin (●), and the photochemical activity of slow rhodopsin (△) are plotted as functions of the length of bleaching.

the flash yield (given as the percent change in transmitted light intensity extrapolated to the time of the flash), is probably due to kinetic complexities of the photochemistry and has been described before for purified purple membrane sheets (Eisenbach et al., 1976; Ort & Parson, 1978; Scherrer et al., 1981). L-33 membranes show more distinctly biphasic kinetics, consisting of a rapid phase of 4.5-ms half-life and a much slower phase (Figure 1B). In this preparation, the two kinetic components are in about equal proportions; in other batches of L-33 envelopes, that ratio of the two varies between 0.8 and 1.2. Bogomolni & Spudich (1982) have shown that in the L-33 membranes the rapid phase corresponds to the photocycle of halorhodopsin, while the slow phase reflects the recovery of slow rhodopsin. The cycling rate for the latter cannot be estimated from our experiments because the flash repetition rate exceeded the recovery rate of this pigment and the bleached intermediate built up during the flash regime. The depletion of the slow rhodopsin during the flashing was not so great, however, as to appreciably decrease the flash yield. Thus, the kinetic analysis illustrated in Figure 1 is adequate to resolve the flash yields for halorhodopsin and slow rhodopsin and to calculate the rate of recovery for halorhodopsin. It should be noted that the photocycle parameters calculated from such experiments were unchanged when gramicidin and uncouplers were added to eliminate the development of any membrane potential and/or pH gradient across the vesicle membranes during the flash regime. We show in Figure 1C the bleaching and recovery traces observed with L-07 membranes, reconstituted in vitro with limiting amounts of retinal, which corresponds virtually exclusively to the photochemical behavior of slow rhodopsin. Indeed, when L-07 vesicles are reconstituted in this way, little or no light-driven chloride transport activity is seen.

Hydroxylamine bleaching of slow rhodopsin was much more rapid than for halorhodopsin, and this procedure could be used to prepare L-33 membranes largely lacking the slow pigment. Figure 2 shows traces of flash-induced absorbance changes after different periods of illumination with hydroxylamine. It is evident that after 6 h of bleaching at pH 7 nearly all of the slow phase is eliminated. The recovery rates are unaffected. The time course of the effects of bleaching on the two kinetic components is also given in Figure 2, together with the activity of the vesicle samples in producing membrane potential upon illumination. The data indicate that under these conditions the photochemical behavior ascribed to halorhodopsin is removed at least 30-fold more slowly than the photocycle of slow rhodopsin. The ability to generate a membrane potential, i.e., to transport chloride, followed the inactivation of the halorhodopsin photochemistry well beyond the times shown in Figure 2 (up to 48 h of bleaching), as expected.

The ability to selectively bleach slow rhodopsin was used to compare the flash yields of halorhodopsin and slow rhodopsin with their relative amounts in the membranes. One sample of L-33 vesicles was partially bleached, and addition of retinal reconstituted the slow rhodopsin. A second sample was completely bleached, and addition of retinal reconstituted both slow rhodopsin and halorhodopsin. Amounts of pigment reconstituted were determined from the amplitudes of the 590-nm bands in the difference spectra (reconstituted vs. nonreconstituted), and flash yields were calculated from photocycle traces, as in Figures 1B and 2. We found that the ratio of fast and slow components of the flash yield in these samples corresponded reasonably well to the calculated ratio of absorption at 590 nm for halorhodopsin and slow rhodopsin, respectively (not shown). Thus, flash yields provide good estimates (Bogomolni & Spudich, 1982) of the relative amounts of these two pigments. Optimal hydroxylamine bleaching, which gave good reconstitution yields for both halorhodopsin and slow rhodopsin, was at pH 6, rather than at pH 5 or 7.

pH Dependence of the Flash-Induced Absorbance Changes for Bacteriorhodopsin, Halorhodopsin, and Slow Rhodopsin. Flash yields and first-order rates of recoveries for the halobacterial pigments in vesicle membranes were determined in experiments similar to those in Figure 1, but at different pH

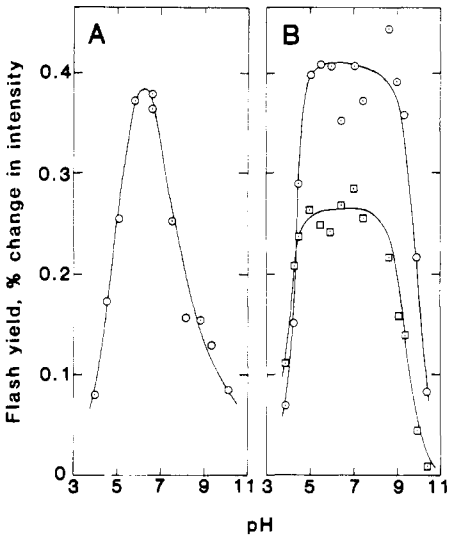

FIGURE 3: pH dependence of the flash yields of bacteriorhodopsin (A), halorhodopsin (B, O), and slow rhodopsin (B, □). Flash-induced absorbance changes were determined and analyzed as in Figure 1. For bacteriorhodopsin, JW-5 membranes reconstituted with retinal were used; for halorhodopsin and slow rhodopsin, L-33 membranes were used, as in Figure 1. Flash yields are expressed as the percent increase in transmitted light intensity due to the photobleaching, calculated for a single flash.

values. Flash yields for bacteriorhodopsin are given in Figure 3A. These were calculated for the rapid component of the traces (Figure 1A), which amounted to about 90% of the total yield near pH 6–7, although only about half at the extreme pH values. This parameter exhibits a narrow peak, centered around pH 6.0. A similar pH dependence was observed for the flash kinetics in isolated purple membrane sheets, suspended in 100 mM NaCl (not shown). These results correspond to those reported by Ort & Parson (1978) on the pH independence of the total flash-induced absorbance changes in bacteriorhodopsin, and also to the pH dependence of the rapid photocycle component apparent from the data of these authors. Unlike with this kinetic component of bacteriorhodopsin, we find that the flash yields of halorhodopsin and slow rhodopsin show little change between pH 5 and 9 (Figure 3B).

The rate of recovery from flash bleaching is also sharply pH dependent in bacteriorhodopsin. The two components (Figure 1A) of the flash traces behave differently: the rate of the major, rapid phase of recovery increases with pH (Figure 4), and the rate of the minor, slow phase decreases with pH. Thus, although in Figure 4 we show the pH dependence of the rapid component, the overall recovery rate of the pigment will decrease with increasing pH. We find that, as reported by Eisenbach et al. (1976), Ort & Parson (1978), and Scherrer et al. (1981), the photocycling of bacteriorhodopsin in isolated purple membrane sheets at lower ionic strength can be described in a similar way, although the relationship of fast and slow components is somewhat altered (not shown). Again in contrast, the pH dependence of the photocycle kinetics of halorhodopsin is much simpler and appears to be nearly independent of proton concentration between pH 5 and 9 (Figure 4).

Effects of Chloride on the Flash-Induced Absorbance Changes in Halorhodopsin. Figure 5 shows flash traces for halorhodopsin plus slow rhodopsin (A) and slow rhodopsin (B), using vesicles suspended in Na_2SO_4 instead of NaCl. The absorbance changes which correspond to the photochemical behavior of slow rhodopsin are virtually unaffected by chloride,

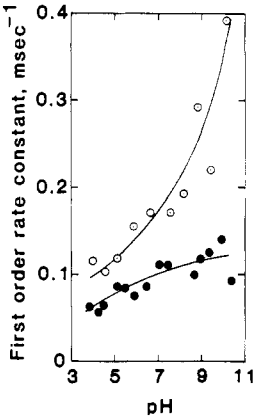

FIGURE 4: pH dependence of the flash bleaching recovery rates of bacteriorhodopsin (O) and halorhodopsin (●). The first-order rate constants were calculated from the same data as in Figure 3.

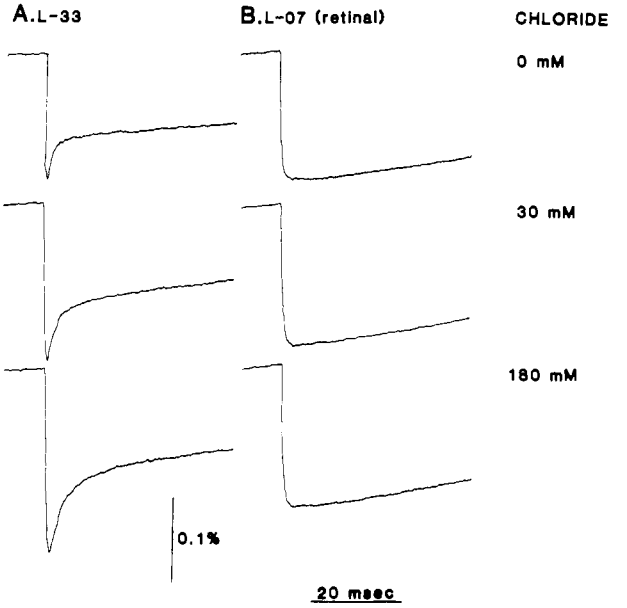

FIGURE 5: Effect of chloride on the flash-induced absorbance changes of halorhodopsin plus slow rhodopsin (A) and slow rhodopsin (B). Strains of *H. halobium* and flash conditions were as described in Figure 1, but the vesicles were suspended in 1.5 M Na_2SO_4, pH 6.5, containing the indicated concentrations of NaCl. Vertical scale shown is for (A); the traces for (B) are enlarged 2×.

as are flash traces obtained for bacteriorhodopsin in envelope vesicles (latter not shown). In contrast, the photocycle of halorhodopsin is seen to be altered at very low chloride concentrations: the flash yield for halorhodopsin is diminished, and the recovery rate is severalfold increased (trace labeled as zero chloride concentration). The bleaching and recovery traces under these conditions correspond to what Bogomolni et al. (1981) earlier described in more detail as the "low salt" scheme of photochemical transitions for halorhodopsin but have recognized since to be altered photochemistry due to the absence of chloride (Weber et al., 1982). Adding sodium chloride restores the characteristic 5–10-ms recovery kinetics (Figure 5A). As shown in Figure 6, a half-maximal increase in flash yield is observed at about 40 mM chloride. Similarly to what we find for the transport properties of halorhodopsin, the anion effect on the photocycle was rather specific. Bromide was effective in replacing chloride, but sulfate, phosphate, and nitrate were not. Similar results were obtained with L-33 membranes suspended in 3 M potassium phosphate upon addition of chloride (not shown). In contrast, the photocycle of slow rhodopsin, determined as the slow component in L-33

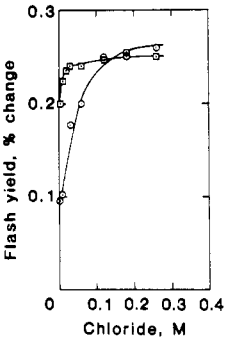

FIGURE 6: Dependence of flash yields for halorhodopsin and slow rhodopsin on chloride concentration. Data calculated from flash bleaching traces, such as shown in Figure 5A, by curve peeling. Flash yields are expressed as the percent increase in transmitted light due to photobleaching, calculated for a single flash. (○) Rapid kinetic component (halorhodopsin); (□) slow kinetic component (slow rhodopsin).

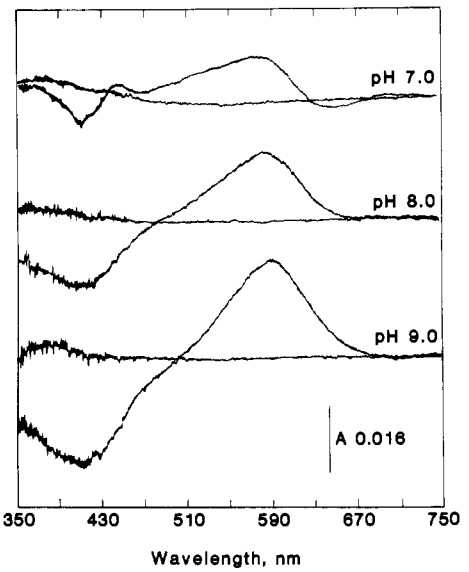

FIGURE 7: Effect of chloride on the absorption spectrum of *H. halobium* strain L-33 membranes. The vesicles were suspended in 1.5 M Na_2SO_4 (5 mg/mL protein), and the pH was adjusted to the indicated values. The samples were placed in two 1 × 1 cm cuvettes, and difference spectra were recorded before and after addition of 4 M NaCl to the sample cuvette to give a final concentration of 200 mM and of 1.5 M Na_2SO_4 of equivalent volume to the reference cuvette.

membranes or the photoactive species in reconstituted L-07 membranes, is only slightly affected by chloride, and the small stimulatory effect observed is complete at a chloride concentration of 30 mM (Figure 6). As expected, L-33 membranes bleached for 6 h with NH_2OH to selectively remove slow rhodopsin exhibited virtually exclusively the chloride-dependent photocycle (not shown).

Effects of Chloride on the Absorption Spectrum of Halorhodopsin. L-33 membranes appeared less colored in Na_2SO_4 than in NaCl. Addition of chloride caused spectroscopic changes in both visible and near-UV regions, as shown in Figure 7. The changes increased in amplitude with pH up to 9; above this pH, no chloride effects could be seen. It is evident in Figure 7 that chloride causes increased absorption at 590 nm and decreased absorption at 410 nm. Figure 8 shows that the amplitudes of the chloride-dependent increase at the higher wavelength and the decrease at the lower wavelength are reciprocal and of about the same magnitude. Between pH 5 and 8, these effects were brought about at chloride concentrations below 200 mM, and half-maximal

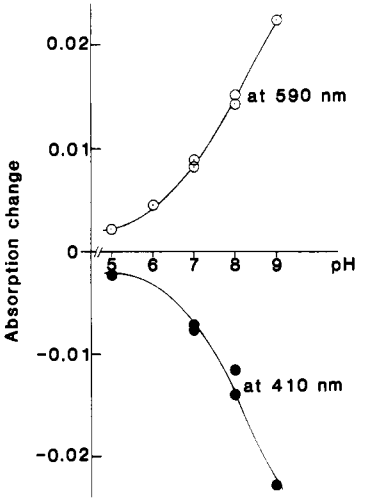

FIGURE 8: Absorption changes at 590 and 410 nm upon addition of chloride to *H. halobium* strain L-33 membranes suspended in 1.5 M Na_2SO_4. Conditions as described under Figure 7.

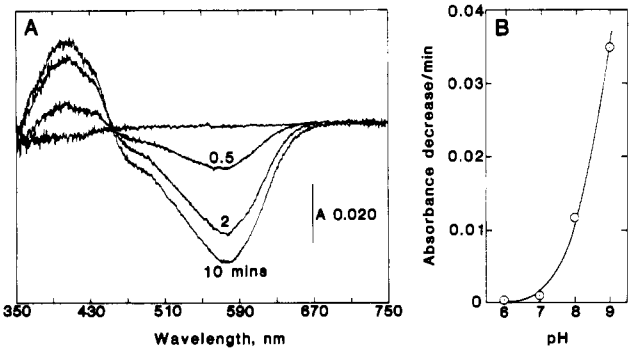

FIGURE 9: Alkaline photoreaction of halorhodopsin. (A) Demonstration of a 580- to 410-nm shift during illumination. L-33 membranes were suspended in 4 M NaCl at pH 9 (5 mg/mL protein) and illuminated with yellow light (OG 530 filter) for the indicated periods of time, followed by determination of difference spectra between these samples and nonilluminated membranes. (B) pH dependence of the initial rate of absorption change at 580 nm during illumination.

effects were observed at 40–50 mM chloride. At pH 9, the apparent affinity constant increased to about 70 mM. At pH 7 and below, additional, minor spectral features are seen near 650 and 430 nm, which we cannot interpret at present.

L-33 membranes, bleached with hydroxylamine for 6 h as in Figure 2 in order or remove slow rhodopsin, did exhibit the spectroscopic changes shown in Figure 7, indicating that of the two pigments in these samples it is halorhodopsin which responds to the chloride. Consistent with this result, reconstituted L-07 membranes did not show the effects, suggesting that slow rhodopsin undergoes no chloride-dependent spectral shifts.

"Alkaline Photoreaction" of Halorhodopsin. When L-33 membranes suspended in 4 M NaCl at pH 9 were illuminated with sustained yellow light at intensities near the level which will saturate the light-driven transport system (Schobert & Lanyi, 1982), the halorhodopsin chromophore was bleached. Figure 9A shows difference spectra between a dark sample and one illuminated for periods up to 10 min. The decrease of absorption near 580 nm is seen to be accompanied by the rise of a new band near 410 nm. The absorption band apparently removed during the illumination is about 10-nm blue shifted from the absorption band of halorhodopsin. This is most probably due to the appearance of another spectral species during the illumination, which absorbs at 630 nm (B. Schobert and J. K. Lanyi, unpublished experiments). The rate

of the photoreaction increased sharply with pH (Figure 9B), but its maximum extent was not pH dependent up to pH 9. Reversal of the change in the dark was slow (hours) and incomplete. The alkaline photoreaction is a property of halorhodopsin only, because after exhaustive illumination as in Figure 9 the flash-induced absorbance changes attributed to halorhodopsin were eliminated, while the flash yield for slow rhodopsin was unchanged. Furthermore, L-33 membranes, partially bleached with hydroxylamine to remove slow rhodopsin, exhibited the alkaline photoreaction, but reconstituted L-07 membranes, which contain mostly slow rhodopsin, did not (results not shown). Ogurusu et al. (1981) described a similar but reversible photoreaction for membranes containing halorhodopsin (and probably also slow rhodopsin) but at much lower salt concentration (0.4 M NaCl).

Halorhodopsin shows a blue shift to about 550 nm in 4 M NaCl when the pH is raised to 10 or above (Lanyi & Weber, 1980). The participation of these species in the alkaline photoreaction described in Figure 9 is unlikely, however, because red light (wavelengths below 610 nm blocked) is effective in producing the spectral shift (not shown). Earlier we suggested that the halorhodopsin content of *H. halobium* membranes can be determined from the blue shift at alkaline pH (Lanyi & Weber, 1980). Since halorhodopsin and slow rhodopsin share this property (B. Schobert and J. K. Lanyi, unpublished results), this method will not distinguish between the two pigments. The alkaline photoreaction described here is specific for halorhodopsin, however, and we recommend illumination in 4 M NaCl at pH 9 and difference spectroscopy as the means to estimate the approximate amount of halorhodopsin in these membranes. By this test, the L-33 membranes used in the present study contain approximately 0.2 nmol of halorhodopsin per mg of protein and, by inference from hydroxylamine bleaching results (see above), about the same amount of slow rhodopsin.

L-33 membranes exhibit the alkaline photoreaction of halorhodopsin also in 1.5 M Na_2SO_4, but even at lower pH (not shown). The amplitude of the spectroscopic changes upon illumination reached a maximum at pH 7 and declined above this pH. The decline is as expected, since in the absence of chloride the 590- to 410-nm shift occurs already in the dark (Figure 7).

Discussion

If halorhodopsin is a light-driven chloride pump, as suggested by recent evidence (Schobert & Lanyi, 1982), tight coupling between the photosensing part of the molecule (i.e., the retinal) and the chloride binding site should give rise to apparent chromophore–chloride interactions, detected as spectroscopic effects of chloride. Two such effects are evident: one on the photochemical behavior of halorhodopsin and another on its absorption spectrum.

The results led to a simplified model for halorhodopsin (HR) (Figure 10) which assumes that the pigment exists in two forms, one with its chloride binding site occupied (HR·Cl) and another with the site vacant (HR). At neutral pH, both of these forms absorb near 590 nm, but they differ in their photocycle pathways. The two kinds of photocycles apparently correspond to the high-salt and low-salt cycles described earlier (Bogomolni et al., 1981) and are characterized with our flash photometer as a 5-ms high-yield photocycle (for HR·Cl) and a 1-ms low-yield photocycle (for HR). Instrumentation which resolves wavelengths (Bogomolni et al., 1981; B. Schobert and J. K. Lanyi, unpublished experiments) reveals the differences in the photointermediates as well. The apparent affinity constant for chloride to produce this effect on the photocycle

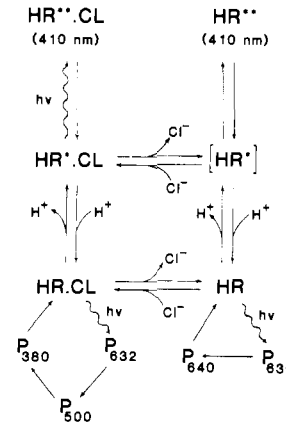

FIGURE 10: Simplified model for the effects of chloride and protons on the chromophore and photochemical behavior of halorhodopsin in *H. halobium* cell envelope vesicles at high ionic strength. HR·Cl represents halorhodopsin with bound chloride; HR, halorhodopsin without chloride; HR*·Cl, deprotonated halorhodopsin with bound chloride; HR*, deprotonated halorhodopsin without chloride; HR**·Cl, photoproduct from halorhodopsin at alkaline pH in the presence of chloride; HR**, blue-shifted form of halorhodopsin, formed spontaneously at alkaline pH in the absence of chloride. The absorption bands of HR·Cl, HR, and HR*·Cl are near 590 nm; that of HR* cannot be measured. Information about the intermediates in the photocycles of HR·Cl and HR is from Bogomolni et al. (1981) ("salt cycle" and "water cycle", respectively).

is about 40 mM, a value similar to what we have observed for all chloride effects on halorhodopsin (Schobert & Lanyi 1982; this study) at high ionic strength, e.g., in 1.5 M Na_2SO_4. Since the interconversion of the two species exhibiting the two kinds of photocycles occur at low chloride concentrations relative to the high total salt concentration, the contribution of chloride to ionic strength plays little or no role in this phenomenon. Reports of a reversible 10–15-nm blue shift in the absorption band of halorhodopsin in the absence of salt (Bogomolni et al., 1981), or specifically chloride ions (Ogurusu et al., 1982), may be significant, but halorhodopsin as a pump is probably denatured at low ionic strength, like other halophilic enzymes (Lanyi, 1974).

The data suggest that the 590-nm absorption band of halorhodopsin is shifted to 410 nm under two different conditions. One of these consists of alkaline pH in the absence of chloride. Th existence of this pathway is deduced from the fact that chloride causes the reappearance of the 590-nm band of halorhodopsin and the disappearance of absorption at 410 nm (Figure 7). This process must be reversible and will take place in the dark. The other pathway occurs upon illumination at alkaline pH even in the presence of chloride (Figure 9) and is poorly reversible. Although at this time a p*K* cannot be assigned to these processes, it is evident from their pH dependence that both pathways require the loss of one or more protons from halorhodopsin. Accordingly, the model in Figure 10 includes the postulated deprotonated species, HR*·Cl and HR*. The visible absorption band of HR*·Cl must be near 590 nm, but that of HR* is uncertain since this species does not accumulate. The photoconversion of HR*·Cl into the 410-nm species (HR**·Cl) is shown in Figure 10, as is the spontaneous conversion of HR* into HR**.

A molecular explanation of the changes illustrated in the proposed model cannot be given because very little is known about the structure of halorhodopsin. In bacteriorhodopsin loss of the retinal Schiff's base proton and trans–cis isomerization will cause a shift similar to the alkaline photoreaction of halorhodopsin, from 568 to 412 nm (Stoeckenius et al., 1979). Indeed, since the protonated Schiff's base absorbs

already near 440 nm in the absence of interaction with the opsin [for model compounds, see Blatz et al. (1972); for bacteriorhodopsin, see Schreckenbach et al. (1977)], any species blue shifted from 440 nm should be strongly suspected to have lost the Schiff's base proton. It seems very likely, therefore, that the shift to 410 nm observed in halorhodopsin is also caused by deprotonation of the Schiff's base. In addition, Ogurusu at al. (1981) demonstrated that, although under conditions somewhat different from ours, the production of the 410-nm species was accompanied by a conversion of the retinal from the trans to the 13-cis form. The observed shifts in the chromophore band must be due to changes in halorhodopsin at the retinal moiety. Conversely, it is evident that little significant change will have occurred near the retinal when its absorption band is unchanged. Hence, we suggest tentatively that the conversion of HR·Cl to HR*·Cl (and possibly that of HR to HR*) reflects deprotonation at a site remote from the chromophore, but the appearance of HR**·Cl and HR** reflects loss of the Schiff's base proton, and perhaps isomerization of the retinal.

The differences in pH dependence between the flash-induced absorbance changes of bacteriorhodopsin and halorhodopsin are readily apparent. The recovery of bacteriorhodopsin from flash bleaching exhibits complex kinetics, which may reflect differences in the behavior of two M intermediates (Hess & Kuschmitz, 1977), conformational changes during the photocycle (Hess & Kuschmitz, 1977; Korenstein & Hess, 1977), or coupling (or decoupling) of the photochemical events to different proton conduction pathways. It seems likely that a pH dependency in the bacteriorhodopsin photocycle reflects requirements for the ionization state of proton donor/acceptor residues. We find that the major kinetic component in the bacteriorhodopsin photocycle Figure 4) and the flash yield for this component (Figure 3A) are highly pH dependent in both envelope vesicles and purple membranes. In contrast, no pH dependence is seen for halorhodopsin in the broad region between pH 5 and 9, either for the bleaching recovery rate (Figure 4) or for the flash yield (Figure 3B). The photochemical events in this pigment are thus found to be nearly completely indifferent to proton concentration at pH values where the light-dependent chloride transport takes place (Schobert & Lanyi, 1982). Hence, in halorhodopsin, the events of the photocycle are not limited by proton transfer between the medium and the protein, as it seems to be in bacteriorhodopsin.

The results in this paper suggest that the chromophore of halorhodopsin communicates poorly with external protons but well with chloride ions. However, in the absence of chloride at alkaline pH, the retinal Schiff's base proton is labilized. The relevance of this to light-driven chloride transport is not certain as yet, but it may provide the basis for further studies of the mechanism of chloride translocation in halorhodopsin.

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Registry No. Chloride, 16887-00-6; hydrogen ion, 12408-02-5.

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