Proton Transport by Halorhodopsin†

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ABSTRACT: In halorhodopsin from *Natronobacterium pharaonis*, a light-driven chloride pump, the chloride binding site also binds azide. When azide is bound at this location the retinal Schiff base transiently deprotonates after photoexcitation with light >530 nm, like in the light-driven proton pump bacteriorhodopsin. As in the photocycle of bacteriorhodopsin, pyranine detects the release of protons to the bulk. The subsequent reprotonation of the Schiff base is also dependent on azide, but with different kinetics that suggest a shuttling of protons from the surface as described earlier for halorhodopsin from *Halobacterium salinarium*. This azide-dependent, bacteriorhodopsin-like photocycle results in active electrogenic proton transport in the cytoplasmic to extracellular direction, detected in cell envelope vesicle suspensions both with a potential-sensitive electrode and by measuring light-dependent pH change. We conclude that in halorhodopsin an azide bound to the extracellular side of the Schiff base, and another azide shuttling between the Schiff base and the cytoplasmic surface, fulfill the functions of Asp-85 and Asp-96, respectively, in bacteriorhodopsin. Thus, although halorhodopsin is normally a chloride ion pump, it evidently contains all structural requirements, except an internal proton acceptor and a donor, of a proton pump. This observation complements our earlier finding that when a chloride binding site was created in bacteriorhodopsin through replacement of Asp-85 with a threonine, that protein became a chloride ion pump.

The bacteriorhodopsins and the halorhodopsins are small integral membrane proteins in various species of halobacteria that, upon illumination, transport protons out of the cells and chloride ions into the cells, respectively (Lanyi, 1990, 1993; Oesterhelt et al., 1992; Rothschild, 1992; Ebrey, 1993). Since the amino acid sequence (Blanck & Oesterhelt, 1987; Lanyi et al., 1990; Otomo et al., 1992; Soppa et al., 1993) and the tertiary structure (Henderson et al., 1990; Havelka et al., 1995) of these proteins are similar, and the ion transport is triggered in both cases by photoisomerization of the *all-trans*-retinal to 13-*cis*-retinal, a shared transport mechanism for protons and chloride has been long regarded as likely (Oesterhelt & Tittor, 1989; Keszthelyi et al., 1990; Oesterhelt et al., 1992).

Under special conditions halorhodopsin from *Halobacterium salinarium* was reported to transport protons. This occurred through a two-photon reaction in which the unprotonated retinal Schiff base was first accumulated by sustained illumination with green light and then photoexcited with blue light (Bamberg et al., 1993). The proton transport was from the extracellular to the cytoplasmic surface, *i.e.*, in the direction opposite from bacteriorhodopsin. It was proposed that the protons were translocated because (a) proton conductive pathways exist between the Schiff base and the two membrane surfaces and (b) the accessibility of the Schiff base to the surfaces changes upon the sequential photoisomerizations. In the 13-*cis* state produced by green light, the access had to be to the cytoplasmic side where the Schiff base proton was released, and in the *all-trans* state produced upon blue-light illumination of the 13-*cis* state the access would be changed to the extracellular side, where a proton was taken up. The transport is very similar to proton transport by bacteriorhodopsin in which Asp-85 is replaced with an asparagine. This recombinant protein does not transport in the usual bacteriorhodopsin mode, *i.e.*, in a single-photon reaction of the protonated Schiff base, because the extracellular proton acceptor is absent (Subramaniam et al., 1990; Kataoka et al., 1994). However, it transports protons in a two-photon reaction (green *plus* blue light) and in the extracellular to cytoplasmic direction as halorhodopsin (Tittor et al., 1994).

The analogy of halorhodopsin and bacteriorhodopsin with respect to chloride transport appears to be somewhat more direct. According to recent evidence (Sasaki et al., 1995), chloride transport replaces proton transport when Asp-85 of bacteriorhodopsin is exchanged for a threonine, the residue found in the halorhodopsins at this location. Chloride-dependent spectral shifts and the photocycles of this mutant bacteriorhodopsin and halorhodopsin from *Natronobacterium pharaonis* (Scharf & Engelhard, 1994; Váró et al., 1995a) suggested that chloride binds initially near both Thr-85 and the Schiff base. The similarities of these single-photon photocycles and the way their steps were affected by changing the chloride concentration, as well as the direction of the translocation, suggested that the mechanism of the chloride transport in D85T recombinant bacteriorhodopsin was analogous to chloride transport by halorhodopsin. Thus, in this case it was the single amino acid replacement, rather than a difference of the nature of the photoreaction, that changed the ion specificity of the transport.

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The observation of chloride transport by bacteriorhodopsin suggested that under suitable conditions halorhodopsin might transport protons in the manner of wild-type bacteriorhodopsin. Demonstration of this would complete the analogy of the two proteins. Introducing aspartate residues into halorhodopsin from *H. salinarium*, at locations where they should function like Asp-85 and Asp-96 in bacteriorhodopsin, did not convert it into a proton pump [mentioned in Havelka et al. (1995)]. Since the pK_a of the Schiff base of halorhodopsin becomes considerably lower after photoisomerization of the retinal to 13-*cis* (Lanyi, 1986), as in bacteriorhodopsin (Govindjee et al., 1994; Brown & Lanyi, 1996), this negative finding means that in the halorhodopsin mutant the engineered aspartate was not a suitable proton acceptor. The reason for this is not clear, since in the absence of transducing protein, sensory rhodopsin I, the third retinal protein of halobacteria which contains an aspartic acid at the position of Asp-85 in bacteriorhodopsin, is a proton pump in both a single-photon cycle (Olson & Spudich, 1993; Bogomolni et al., 1994) and a two-photon cycle (Haupts et al., 1995). We have therefore examined another alternative. Azide catalyzes the light-dependent deprotonation and reprotonation of the Schiff base in halorhodopsin (Hegemann et al., 1985; Lanyi, 1986; Scharf & Engelhard, 1994), and the reprotonation of the Schiff base in the photocycle of the D96N mutant of bacteriorhodopsin where this process is otherwise very slow (Tittor et al., 1989; Otto et al., 1989; Cao et al., 1991). The proton transport reactions in bacteriorhodopsin and halorhodopsin with unprotonated Schiff base were enhanced by azide also (Bamberg et al., 1993; Tittor et al., 1994; Kataoka et al., 1994; Dickopf et al., 1995). The dependence of protonation and deprotonation of the Schiff base of halorhodopsin on the pK_as of various weak acids suggested that the mechanism for the catalysis was a shuttling of protons between the Schiff base and the cytoplasmic surface (Lanyi, 1986). The azide therefore did not traverse the entire membrane and could not facilitate net proton translocation.

Thus, under some conditions azide can act as both a proton acceptor and a donor to the Schiff base. Unlike in the case of halorhodopsin in *H. salinarium*, a blue shift of the absorption maximum upon adding chloride to the halorhodopsin to *N. pharaonis* indicated that this protein contains a high-affinity binding site for chloride near the Schiff base. We had argued from indirect evidence that this site is to the extracellular side of the Schiff base (Váró et al., 1995a,b). Site-specific mutagenesis of halorhodopsin from *H. salinarium* identified an arginine near this location as essential for chloride binding (Rüdiger et al., 1995). Could azide bound to this site function in *N. pharaonis* halorhodopsin like Asp-85 in bacteriorhodopsin? In the present report we demonstrate that azide bound to the chloride binding site functions as an acceptor of the Schiff base proton during the photocycle and that, after deprotonation of the Schiff base, protons are released to the extracellular surface. Another molecule of azide then shuttles protons from the cytoplasmic surface to the unprotonated Schiff base, as in *H. salinarium* halorhodopsin. The result is active electrogenic proton transport in the cytoplasmic to extracellular direction. Thus, azide converts *N. pharaonis* halorhodopsin into a light-driven proton pump analogous, with respect to both the direction and the internal mechanism of the proton translocation, to wild-type bacteriorhodopsin.

MATERIALS AND METHODS

Membrane fragments enriched for halorhodopsin were prepared from *H. salinarium* containing a plasmid vector with the structural genes for either *H. salinarium* or *N. pharaonis* halorhodopsin and the *bop* promoter by the method described before (Váró et al., 1995a). The T126D mutant was constructed by conventional methods, using this vector. All samples contained Na_2SO_4, with or without NaCl, so as to keep the $[Na^+]$ at 2 M. Stationary spectra were measured with a Shimadzu 1601 spectrophotometer connected to a desktop computer.

Absorption changes were followed after photoexcitation with a Nd−YAG laser pulse (532 nm, 7 ns), as in numerous earlier publications of ours [*e.g.*, Váró et al. (1995c)]. Transient pH changes during the photocycle were followed with the indicator dye pyranine[1] (Grzesiek & Dencher, 1986). The temperature was regulated at 20 °C throughout.

Cell envelope vesicles were prepared by the method described before (Lanyi & MacDonald, 1979). Transport was measured at about pH 6 in 1.5 M Na_2SO_4, with or without NaCl or K_2SO_4, so as to keep the $[Na^+]$ *plus* $[K^+]$ at 3 M. In transport assays, transmembrane electrical potential was measured with a TPP^+ electrode (Shinbo et al., 1978) and proton extrusion or uptake with a pH electrode as in Váró et al. (1995a).

RESULTS

Binding of Chloride and Azide by Halorhodopsin. The binding of chloride to halorhodopsin from *N. pharaonis* can be detected through a blue shift of the absorption maximum (Scharf & Engelhard, 1994; Váró et al., 1995a). Figure 1A shows difference spectra that reveal a simple two-state equilibrium, dependent on the binding of chloride. Essentially the same difference spectra, with an isosbestic point at 602 nm, were obtained with azide (not shown), suggesting that azide binds to the same site as chloride. In Figure 1B the amplitudes of the difference spectra are plotted as functions of chloride or azide concentrations. The apparent dissociation constant for the chloride complex is 1.7 mM and for the azide complex 10 mM, consistent with earlier values (Scharf & Engelhard, 1994; Váró et al., 1995a). As shown below, proton concentration does not change the calculated dissociation constants of either chloride or azide. Since the pK_a of azide is 4.7 and therefore above pH 6 $[N_3^-] \cong [\text{azide}]_{\text{total}}$ and $[HN_3]$ varies linearly with $[H^+]$, this pH independence implicates the anionic form of azide in the binding and the spectral shift.

If the anion binding is near the retinal Schiff base, as suggested by the blue shift of the absorption band, it can be expected to elevate the pK_a of the protonated Schiff base through coulombic effects and/or possibly through hydrogen bonding. Spectroscopic titrations of the protonation state of the Schiff base, utilizing the shift of the absorption maximum from about 580 to 410 nm upon deprotonation, were carried out in Na_2SO_4 and in various mixtures of sulfate and NaCl or sodium azide. A series of such pH-dependent different spectra, but in sulfate alone, is given in Figure 2A.

[1] Abbreviations: TPP^+, tetraphenylphosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; Bis-tris propane, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate.

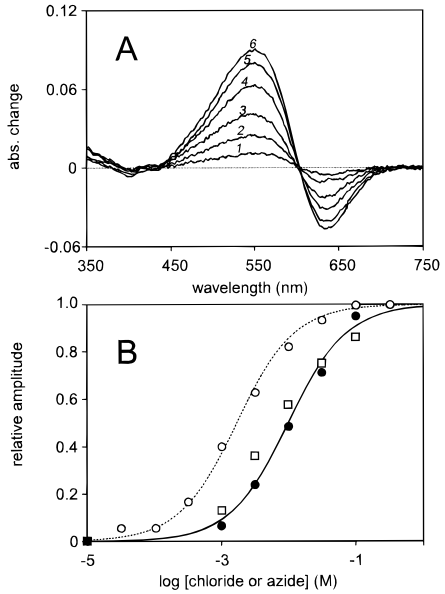

FIGURE 1: Binding of chloride and azide to halorhodopsin. (A) Spectroscopic titration of membranes with NaCl in Na_2SO_4. Chloride plus sulfate minus sulfate difference spectra 1−6 with chloride concentrations of 1, 3, 10, 30, 100, and 300 mM. Total [Na^+] was kept at 2 M, buffered at pH 7.0 with 50 mM phosphate. (B) Titration curves based on the blue shift of the absorption maximum in panel A, upon adding chloride (○, dashed line) or azide (●, solid line), and the amplitude of light-induced absorption change at 410 nm (□) as in Figure 3A.

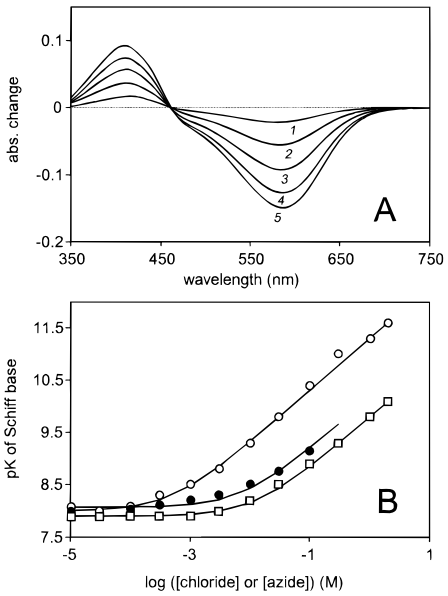

FIGURE 2: Effects of chloride and azide on the pK_a of the protonated Schiff base. (A) Deprotonation of the Schiff base of *N. pharaonis* halorhodopsin in 1 M Na_2SO_4 upon raising the pH. Higher pH minus pH 6.0 difference spectra 1−5 are given for pH 7.5, 8.0, 8.5, 9.0, and 9.5. Buffer was 25 mM CAPS plus 25 mM Bis-tris propane. (B) pK_a of the Schiff base, calculated from spectra such as in panel A, as a function of chloride (○) or azide (●) in *N. pharaonis* halorhodopsin, or chloride (□) in *H. salinarium* halorhodopsin.

The pK_a values obtained for halorhodopsin from *N. pharaonis* from these titrations are shown in Figure 2B as functions of the chloride or azide concentration (open and closed circles). The limiting pK_a in the absence of chloride or azide is 7.9−8.0. In the presence of added anion the apparent pK_a is significantly raised, and to diffferent extents with chloride and azide. The kinetic model that uniquely describes the data is

$$-C{=}N + H^+ \leftrightarrow -C{=}NH^+ + A^- \leftrightarrow -C{=}NH^+\,A^-$$

where −C═N and −C═NH^+ represent the unprotonated and protonated retinal Schiff base, respectively, and A^- is the anion (chloride, or azide at pH well above its pK_a). Thus, chloride or azide binding depends on the positive charge of the protonated Schiff base. The dissociation constant in the best fit of this model (lines in Figure 2B) is 0.5 mM for chloride and 8 mM for azide, consistent with the values from the spectral shifts of the chromophore.

Figure 2B contains this kind of data also for halorhodopsin from *H. salinarium,* in the presence of chloride. Although the detergent-solubilized protein behaved differently with respect to pH and chloride (Schobert & Lanyi, 1986), the pK_a of the Schiff base in the membrane-bound protein from this species varies with chloride concentration similarly to the *N. pharaonis* protein. It appears that while the absorption maximum of the *H. salinarium* protein shifts toward the red rather than toward the blue upon chloride binding (Schobert et al., 1983; Steiner & Oesterhelt, 1983; Steiner et al., 1984), suggesting that the binding site is different from that in the *N. pharaonis* protein, the binding of the chloride is near enough to the Schiff base to depend on its protonation state. The nature of the difference in the binding sites in the halorhodopsins from the two species is not yet clear, and the residues in the vicinity of the Schiff base provide no obvious clues. The results show further that at the physiological chloride concentration of several molar the pK_a of the Schiff base in the *N. pharaonis* protein is raised to above 11.5, *i.e.*, far above that of the *H. salinarium* protein. This is in keeping with the fact that the pH of the natural environment of *N. pharaonis* cells is above 10 (Tindall et al., 1980), while *H. salinarium* grows near neutral pH.

Below pH about 7.5 no significant deprotonation of the Schiff base occurred in the unphotolyzed proteins, in either sodium sulfate alone or with added chloride. While sustained illumination accumulated a state with unprotonated Schiff base that reprotonated on the tens of minutes time scale as described before (Lanyi & Schobert, 1983; Hegemann et al., 1985), up to pH 7.5 its amount in the *H. salinarium* protein was no more than about 10% of the total, and less in the *N. pharaonis* protein (not shown). All the experiments described below were with halorhodopsin from *N. pharaonis*, and the decrease of the amount of protein with protonated Schiff base was not significant under any of the conditions used.

Deprotonation of the Schiff Base upon Flash Illumination. Absorption changes at 410 and 590 nm after pulse photoexcitation of halorhodopsin in Na_2SO_4 revealed that virtually no deprotonation of the Schiff base (absorption increase at 410 nm) occurred in the absence of azide (Figure 3A). With azide concentrations between 1 and 100 mM, however, such a state, analogous to the M photointermediate of bacteriorhodopsin, did arise, and in significant amounts relative to the depletion signal at 590 nm (Scharf & Engelhard, 1994; Figure 3). At 100 mM azide the ratio of depletion at 590 nm to absorption rise at 410 nm was 1.5, a value not very different from 1.7, the ratio calculated for 100% conversion to the deprotonated Schiff base in the bacteriorhodopsin photocycle (Zimányi & Lanyi, 1993). Thus, the transient deprotonation of the Schiff base in halorhodopsin under these

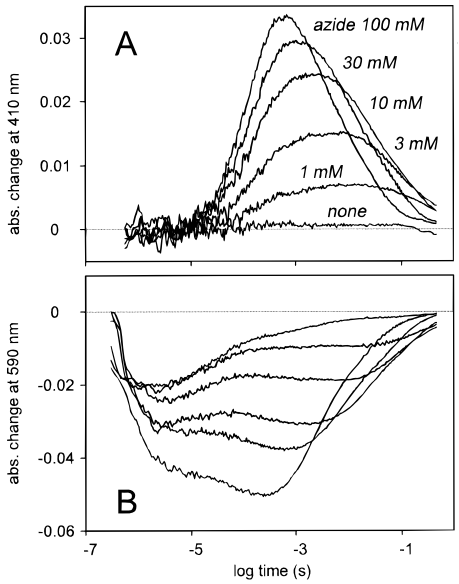

FIGURE 3: Flash-induced transient absorption changes in the presence of azide. (A) The appearance of deprotonated Schiff base is detected at 410 nm in 1 M Na_2SO_4, pH 7.0, with azide concentrations 0, 1, 3, 10, 30, and 100 mM, as indicated. (B) The corresponding absorption changes at 590 nm, at the azide concentrations indicated in panel A in the direction of increasing negative amplitudes.

conditions is extensive. Analysis of the traces in Figure 3A indicated that the time constant for the rise of this M-like state was nearly independent of azide but its amplitude was strongly azide-dependent. Figure 1B includes also the amplitudes of the absorption increase at 410 nm as functions of azide concentration. They nearly coincide with the amplitudes of the spectral shifts. The correlation between the appearance of the M-like state in the photocycle and the spectral shifts in the unphotolyzed protein suggests that the deprotonation of the Schiff base depends on the prior binding of the anionic azide near the Schiff base. It seems likely, therefore, although not proven by these results, that the azide bound at this location is the acceptor of the Schiff base proton.

Unlike the rate of the rise, the rate of the decay of the absorption at 410 nm is strongly dependent on the concentration of azide (Figure 3A). This dependence is linear with azide concentration up to at least 100 mM. The catalysis of the reprotonation of the Schiff base by azide is therefore *not related* to the binding of azide detected by spectral shifts that saturates with a binding constant of 10 mM (Figure 1B). It must be based on a different mechanism. Figure 4 shows the absorption changes at 410 nm at different pH values between 5.5 and 7.0. Again, the rise and the decay behave differently. The deprotonation of the Schiff base is pH-independent, while its reprotonation is strongly accelerated when the pH is lowered.

The azide dependence of the M-like state in these experiments implicates binding of azide at the anion site that also can be occupied by chloride. Figure 5A shows that, as expected, 10 mM azide is ineffective in the presence of 30 mM chloride. Increasing the concentration of azide to 300 mM partly overcomes this inhibition (Figure 5B), suggesting that azide and chloride compete for the same site but deprotonation of the Schiff base will occur only when azide is the bound anion.

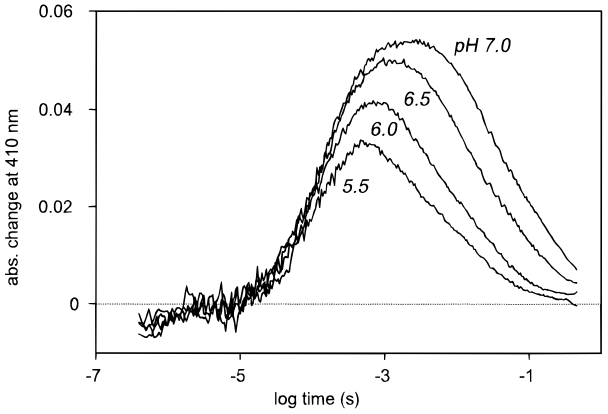

FIGURE 4: Flash-induced deprotonation of the Schiff base as a function of pH. Experiments were performed as in Figure 3A, but at 10 mM azide and the pH indicated.

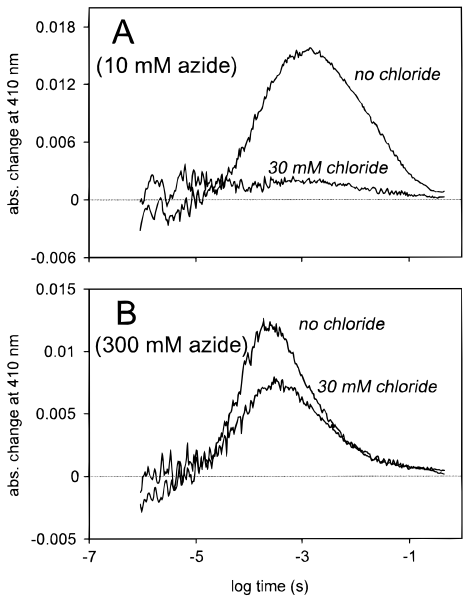

FIGURE 5: Competition of azide and chloride for binding. Flash-induced deprotonation of the Schiff base was followed as in Figure 3A, at different chloride concentrations with 10 mM azide (A) or 300 mM azide (B).

Transient Proton Release in the Presence of Azide. The observed deprotonation of the Schiff base suggests that, as in bacteriorhodopsin, under these conditions a proton might be released in the halorhodopsin photocycle also. Figure 6 shows the absorption increase at 410 nm that originates from deprotonation of the Schiff base, and the net absorption change at 457 nm (with pyranine minus without pyranine) that detects pH change in the bulk (Grzesiek & Dencher, 1986). The latter indicates that protons are transiently released from the protein after deprotonation of the Schiff base. The observed proton release is delayed relative to the release to the surface, probably because buffering groups at the surface delay the transit of the protons to the bulk, as demonstrated for bacteriorhodopsin (Heberle & Dencher, 1992). The amplitude of the pH decrease relative to the amplitude of the absorption rise at 410 nm is about half that in bacteriorhodopsin, probably for the reason that, unlike purple membranes, the membranes used here contained significant amounts of proteins other than halorhodopsin and these would contribute buffering. Another alternative would be that not all of the protons from the Schiff base are released to the surface. Because of its lesser amplitude, and because

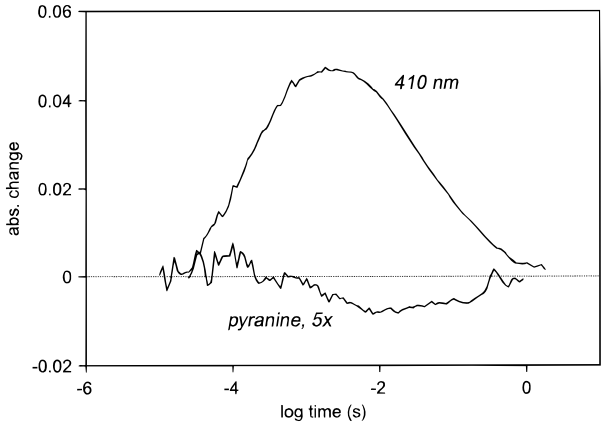

FIGURE 6: Transient proton release and uptake after photoexcitation in the presence of 10 mM azide. The trace at 410 nm shows deprotonation of the Schiff base as in Figure 3A. The trace labeled as pyranine reflects pH change in the bulk. Controls (not shown) include wild-type bacteriorhodopsin, which revealed no significant buffering by azide at the concentration used, and a halorhodopsin sample without azide that revealed no pyranine signal.

the quantum yield of the photoisomerization of halorhodopsin is about half that of bacteriorhodopsin (Lanyi, 1984; Oesterhelt et al., 1985), the pyranine signal was more difficult to detect than in the other protein. Inasmuch as the signal/noise ratio permits estimation of the time constant of the subsequent proton uptake in Figure 6, it correlates with the absorption decrease at 410 nm. No pH change was observed in the absence of azide (not shown).

Transport Activity in the Presence of Azide. Cell envelope vesicles prepared from halobacteria (Lanyi & MacDonald, 1979) can be used to assay light-driven ion transport activity, through measurement of either membrane potential with a TPP^+ electrode (Shinbo et al., 1978) or the appearance or disappearance of protons in the medium with a glass electrode (Lanyi & Oesterhelt, 1982). The former is a sensitive method for detecting electrogenic transport but does not identify the transported ion, while the latter can distinguish between active proton transport (short-circuited by protonophores but enhanced by abolishing the transmembrane electrical potential) and the transport of other species when the latter is electrogenic and therefore results in passive proton movement (enhanced by protonophores but eliminated by abolishing the transmembrane electrical potential).

Illumination of envelope vesicles containing *N. pharaonis* halorhodopsin in the presence of chloride produced a large interior negative electrical potential from the inward chloride transport and an accompanying passive proton uptake (Duschl et al., 1990; Váró et al., 1995a). In Na_2SO_4 a residual small but reproducible pH change upon illumination had suggested that, although much less effectively, SO_4^{2-} or HSO_4^- is also transported. Figure 7 shows the amplitudes of transmembrane electrical potential measured with a TPP^+ electrode under these conditions, but as functions of azide concentration. The uptake of TPP^+ indicated that in sulfate a small negative interior electrical potential develops, consistent with the passive proton influx reported earlier. With added azide the amplitude of this electrical potential increased, suggesting that additional electrogenic transport occurs. However, above 2 mM azide the amplitude declined again. The reason for this is evident from the observed effect of azide on the electrical potential from chloride transport (Figure 7). Significant competition of azide for the chloride

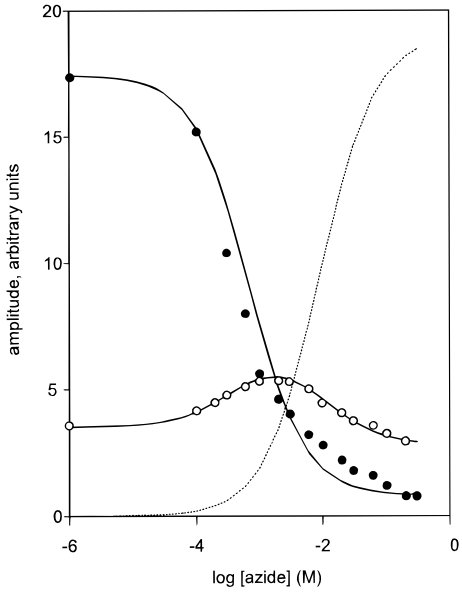

FIGURE 7: Azide-dependent electrogenic transport upon illumination of halorhodopsin. Cell envelope vesicles containing *N. pharaonis* halorhodopsin in 1.5 M Na_2SO_4 were illuminated with continuous yellow light (>530 nm). Light-dependent transmembrane electrical potential was measured with a TPP^+ (tetraphenylphosphonium) electrode in the absence of chloride (○) or its presence in 1.35 M Na_2SO_4 plus 0.3 M NaCl (●). In the measured range the electrode response is roughly linear with the transmembrane electrical potential in the vesicles. Dashed line is the calculated effect of azide without its protonophoric activity, with the response from sulfate subtracted.

binding site in the protein would not occur at the chloride concentration of 300 mM used. However, as is well-known, azide will abolish transmembrane electrical potential as a protonophore and/or membrane-permeant anion because both its protonated and anionic forms will cross the membranes. The data in sulfate plus azide were corrected for this effect, using a simple pump and leak model in which the leak is linearly dependent on the amount of accumulated charges in the vesicles and defined by the parameters of the inhibition in the control curve in Figure 7. This calculation produced the curve shown with a dotted line. If it represents the electrical potential that would be created without the protonophoric effect of azide, the azide-dependent electrogenic transport is comparable in extent to the chloride transport. The apparent binding constant for azide is then 9 mM. This value is consistent with the dissociation constant for the binding of azide, from both spectral shift and the deprotonation of the Schiff base (Figure 1B). These results therefore correlate the electrogenic transport with the binding of the azide and the azide-dependent deprotonation of the Schiff base in the photocycle, but they do not identify the transported ion.

Measurements of illumination-dependent pH change in suspensions of these vesicles in 1.5 M sodium sulfate, under the conditions used in Figure 7, demonstrated that, depending on the vesicle preparation, 7−20 protons/halorhodopsin were taken up (not shown). This is expected to occur in response to the interior negative electrical potential that develops from sulfate transport. Added at 5 μM, the protonophore CCCP somewhat increased the amplitude of this passive proton influx and caused it to be more rapid, also as expected. After addition of 2 mM azide, however, the proton uptake changed to proton extrusion (not shown), in spite of the fact that under

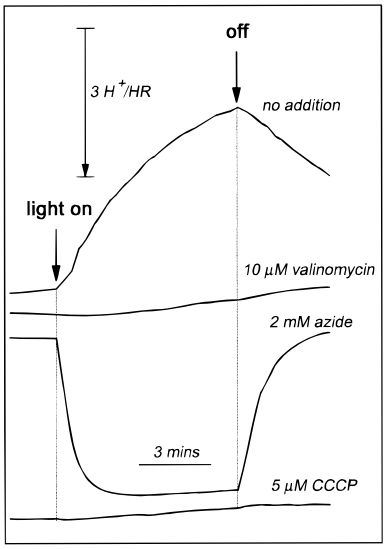

FIGURE 8: Azide-dependent active transport of protons upon illumination of halorhodopsin. Suspensions of cell envelope vesicles containing *N. pharaonis* halorhodopsin were assayed in 1.3 M Na_2SO_4 *plus* 0.2 M K_2SO_4 for light-dependent pH changes. The indicated additions were cumulative.

these conditions the interior negative electrical potential increases (Figure 7). Whether this extrusion represents active proton transport, as its direction suggests, was better studied with the transmembrane potential clamped at zero by equilibrating the vesicles with 1.3 M Na_2SO_4 plus 0.2 M K_2SO_4 and adding the K^+ ionophore valinomycin. Under these conditions any possibility for passive proton transport was removed. Indeed, as shown in Figure 8, the light-dependent passive proton influx was entirely eliminated upon addition of 10 μM valinomycin. After addition of 2 mM azide to this mixture, illumination caused rapid and extensive proton extrusion that was sensitive to the protonophore CCCP (Figure 8). This result demonstrates unequivocally that the azide-dependent interior negative electrical potential in Figure 7 originates from active, outward-directed proton transport. Inward transport of azide, while consistent with the increased electrical potential, would have produced increased passive proton uptake and not when the electrical potential was clamped at zero. Outward transport of azide is inconsistent with the sign of the electrical potential. It would have caused the observed proton extrusion, but not with the electrical potential kept at zero. In neither case would CCCP abolish the pH change.

Properties of T126D Halorhodopsin. The threonine residue in halorhodopsin from *N. pharaonis* at the position of Asp-85 in bacteriorhodopsin (Thr-126) was replaced with an aspartate. Such a recombinant protein, constructed from halorhodopsin in *H. salinarium* and also containing an aspartate equivalent to Asp-96 in bacteriorhodopsin, did not transport protons [mentioned in Havelka et al. (1995)]. The absorption maximum of the T126D mutant was blue shifted by 40 nm relative to wild type in 1.5 M sulfate. Presumably, the anionic aspartate near the Schiff base shifts the absorption band in the same manner as chloride or azide bound to the wild-type protein. Lowering the pH, between 7 and 2, did not result in a red shift, as in bacteriorhodopsin, that would indicate that the aspartate becomes protonated (not shown). Possibly, the pK_a of Asp-126 is lower than the pK_a of its equivalent in bacteriorhodopsin. Interestingly, however, the mutant protein was found to be stable at acid pH, unlike the

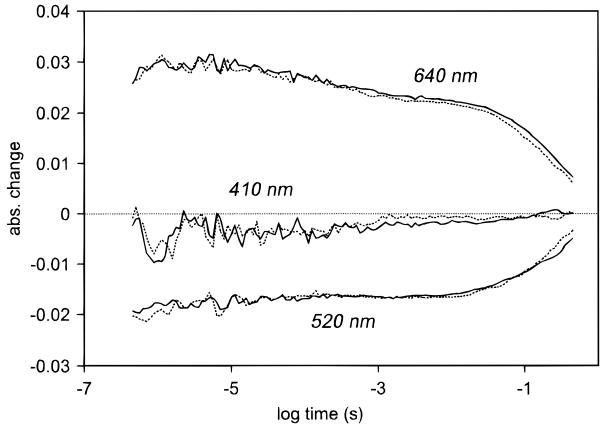

FIGURE 9: Photocycle of T126D halorhodopsin in 1 M Na_2SO_4. Flash-induced transient absorption changes are shown at 640, 410, and 520 nm. Buffer was 50 mM Bis-tris propane at pH 7.0. Solid lines, no azide; dashed lines, 100 mM azide.

wild-type halorhodopsin that irreversibly denatures below pH 5. In experiments similar to those in Figure 1, chloride (up to 2 M) or azide (up to 100 mM) did not shift the absorption maximum of the mutant, suggesting that the anion binding site is abolished by the threonine to aspartate replacement.

Significantly, as evident from Figure 9, no deprotonation of the Schiff base occurs in the photocycle of the T126D protein. The absorption changes are essentially the same as in the wild-type protein in sulfate (Váró et al., 1995a). Unlike the wild-type protein, however, the absorption changes in the photocycle of the mutant did not change in 2 M NaCl (not shown). Furthermore, lack of absorption change at 410 nm indicated that no deprotonation of the Schiff base occurred in the presence of 100 mM azide, in either sulfate (Figure 9) or chloride (not shown). Evidently, the engineered aspartate prevents the binding of azide, but a low pK_a, or an unfavorable geometry relative to the Schiff base, does not allow it to act as proton acceptor. Lack of pH change upon illumination of cell envelope vesicles containing T126D halorhodopsin in the presence of K^+ and valinomycin (not shown) confirmed that the threonine to aspartate mutation does not confer proton transport activity on this protein.

DISCUSSION

We had found earlier that the ionic specificity of bacteriorhodopsin changed from protons to chloride when the proton acceptor residue Asp-85 was replaced with a threonine (Sasaki et al., 1995). In this mutant, chloride was transported in the extracellular to cytoplasmic direction, as in halorhodopsin. Thus, we concluded that bacteriorhodopsin contains all structural properties necessary for chloride transport, except an anion binding site on the extracellular side of the Schiff base. Does halorhodopsin contain, in the same way, all structural attributes that are necessary to transport protons in the cytoplasmic to the extracellular direction, except for a proton acceptor? We report results demonstrating that when an appropriate proton donor (and acceptor) is provided to the Schiff base, halorhodopsin will indeed transport protons in the way bacteriorhodopsin does.

In the presence of the weak acid azide, the photocycle of the *N. pharaonis* halorhodopsin is changed so as to include the deprotonation and the subsequent reprotonation of the

retinal Schiff base (Scharf & Engelhard, 1994; Figures 3−5) and the release and the subsequent uptake of protons (Figure 6). These proton exchange reactions must occur in a vectorial fashion because their consequence is the active electrogenic transport of protons from the cytoplasmic to the extracellular direction (Figures 7 and 8). The observations related to the binding of azide and the kinetics of the protonation reactions in the photocycle suggest a mechanism for the transport. Azide appears to bind to the chloride binding site of this protein that is near the retinal Schiff base, and the azide dependence of the light-induced deprotonation of the Schiff base correlates with the dissociation constant of this binding (Figure 1B). The location of this anion binding site is not firmly identified, but consideration of the residues that are in the vicinity of the Schiff base (Blanck & Oesterhelt, 1987; Lanyi et al., 1990) and the phenotype of a mutant with replaced arginine in this region (Rüdiger et al., 1995) suggest that it is to the extracellular side of the Schiff base. Lack of chloride and azide binding in the T126D mutant are consistent with the extracellular location of the site. Such a location is suggested further by the chloride-dependent spectroscopic changes in the photocycles of this halorhodopsin (Váró et al., 1995b) and D85T bacteriorhodopsin (Sasaki et al., 1995) in the context of the extracellular to cytoplasmic direction of the chloride transport, and the observation that the chloride-binding affinity of D85S is greatly different from that of D85T bacteriorhodopsin.[2] We had considered three possibilities for how the azide bound to this site could facilitate deprotonation of the Schiff base. *First*, the azide anion could be translocated like chloride from the extracellular to the cytoplasmic surface but transiently protonated at the same time from the Schiff base. The subsequent reprotonation of the Schiff base from the cytoplasmic side would result in the net translocation of N_3^-. This alternative is ruled out by the observations in Figure 8 that demonstrate active transport of protons rather than of azide. Reprotonation of the Schiff base from the extracellular rather than the cytoplasmic side would result in the net translocation of HN_3. This is ruled out by the fact that the transport is electrogenic (Figure 7). *Second*, the bound azide could facilitate deprotonation of the Schiff base but with the proton passing through a hydrogen-bonded chain to the cytoplasmic direction, as suggested for bacteriorhodopsin (Le Coutre et al., 1995). This would produce the observed transient proton release to the bulk (Figure 6) but would not result in the negative inside electrical potential (Figure 7) or proton transport in the cytoplasmic to extracellular direction (Figure 8). In any case, the azide binding site in *N. pharaonis* halorhodopsin must be different from the proposed site in bacteriorhodopsin (Le Coutre et al., 1995) because unlike in halorhodopsin (Figure 1A) no spectral shifts can be seen in bacteriorhodopsin when azide (or chloride) is added (not shown). *Third*, the azide anion could be the acceptor for the Schiff base proton in the photocycle, analogous to Asp-85 in bacteriorhodopsin. In this alternative, azide itself cannot be transported because once protonated it is no longer an anion. The pH changes observed with pyranine argue that after protonation the azide either promptly releases the proton to the extracellular surface or the HN_3 is lost from the binding site and dissociates to H^+ and N_3^- in the bulk. This would be unlike Asp-85 in bacteriorhodopsin, which retains the proton until the end of the photocycle but causes instead proton release from Glu-204 (Brown et al., 1995; Richter et al., 1996). On the other hand, halorhodopsin does not contain a protonatable residue at the location equivalent to Glu-204. The third alternative explains all of the results.

The subsequent reprotonation of the Schiff base in the photocycle is also facilitated by azide, but similarly to how azide functions in *H. salinarium* halorhodopsin (Lanyi, 1986) and in the D85N/D96N bacteriorhodopsin mutant (Kataoka et al., 1994) that do not contain suitable extracellular binding sites, *i.e.*, merely by shuttling protons between the Schiff base and the cytoplasmic surface. Shuttling is suggested by the observation of nonsaturating kinetics (at least up to 100 mM), inconsistent with the binding constant of 10 mM for the azide (Figure 3A). This distinguishes it from the deprotonation process. Reprotonation by a shuttling reaction would be either by uptake of HN_3 from the bulk or by H^+ uptake followed by conduction of the proton from the membrane surface to the Schiff base via N_3^- bound with low affinity. The pH dependence of the reprotonation (Figure 4) rules out the alternative in which the proton donor is the bound HN_3 produced during the deprotonation of the Schiff base. The finding of active electrogenic proton transport in the cytoplasmic to the extracellular direction requires unequivocally that if azide is the proton acceptor to the Schiff base from the extracellular side, as strongly suggested by the results, then it must be from the cytoplasmic side that it acts as proton donor. Thus, it appears that the azide molecules at these two locations are functionally equivalent to Asp-85 and Asp-96, respectively, of bacteriorhodopsin.

The results in this report, demonstrating active proton transport by halorhodopsin, complement the earlier finding of chloride transport by bacteriorhodopsin (Sasaki et al., 1995). In both cases a binding site for the unphysiologically transported ion was changed or added, but the photoreactions and conditions under which the transport of the physiologically transported ion would occur were unaltered. We conclude therefore that *the ion specificities of bacteriorhodopsin and halorhodopsin reside in a single binding site rather than in the structure of the ion-conductive pathways*. Likewise, the mechanism of the reorientation switch that confers directionality to the translocation must be common to the two kinds of transport and not dependent on the kind of ion transported. It must be inherent in the common structure of these proteins.

[2] L. S. Brown, R. Needleman, and J. K. Lanyi, unpublished results.

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