Interaction of Proton and Chloride Transfer Pathways in Recombinant Bacteriorhodopsin with Chloride Transport Activity: Implications for the Chloride Translocation Mechanism†

Leonid S. Brown,‡ Richard Needleman,§ and Janos K. Lanyi*,‡

Department of Physiology & Biophysics, University of California, Irvine, California 92717, and Department of Biochemistry, Wayne State University, Detroit, Michigan 48201

Received September 11, 1996; Revised Manuscript Received October 24, 1996⊗

ABSTRACT: When the protonated retinal Schiff base dissociates in the photocycle of the proton pump bacteriorhodopsin, asp-85 is the proton acceptor. Replacing this residue with threonine confers halorhodopsin-like properties on the protein, including chloride transport [Sasaki, J., Brown, L. S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R., & Lanyi, J. K. (1995) *Science 269*, 73−75]. However, the electrostatic interaction between the vicinity of residue 85 and glu-204, a residue located about 10 Å away near the extracellular surface, that is a part of the proton transport mechanism, should still exist. We find that in the D85T mutant glu-204 becomes protonated when chloride is added. This indicates that the binding of chloride at thr-85 must be equivalent to deprotonation of asp-85. The protonation state of glu-204 reports therefore on the presence or absence of chloride bound at thr-85. During the chloride-transport cycle of D85T, but not D85T/E204Q, fluorescein and pyranine detect the transient release of protons from the protein to the surface and the bulk. The release and the subsequent uptake of the protons occur during the rise and decay of a red-shifted photointermediate, respectively, and confirm the earlier suggestion that this state has the same role in the chloride transport as the M intermediate in the proton transport. Consistent with the red-shift of the absorption maximum, the chloride bound near the Schiff base had already moved away, presumably to be released at the cytoplasmic surface, but another chloride ion has not yet been taken up from the extracellular surface. The switch of the connectivity of the chloride binding site from the cytoplasmic to the extracellular membrane surface must occur therefore during the lifetime of this photointermediate.

Halobacterial cell membranes contain two ion pumps based on photoisomerization of *all-trans* retinal to 13-*cis*, the outward-directed proton pump bacteriorhodopsin [for reviews, see Mathies et al. (1991), Oesterhelt et al. (1992), Lanyi (1993), and Ebrey (1993)] and the inward-directed chloride ion pump halorhodopsin [for reviews, see Lanyi (1990) and Oesterhelt (1995)]. The fundamental difference that accounts for the ion specificities and the directions of the transport in the two proteins is the side chain of residue 85, located in the immediate vicinity of the protonated retinal Schiff base. In bacteriorhodopsin this residue is an anionic aspartate that is both the main component of the counter-ion to the Schiff base and the proton acceptor in its deprotonation in the transport cycle. In halorhodopsin it is a threonine that is presumed to be hydrogen-bonded to a chloride ion bound near the Schiff base in the extracellular region. This is likely to be the chloride ion that is translocated during the photocycle. The recombinant D85T[1] bacteriorhodopsin thus behaves like halorhodopsin: it binds chloride near the Schiff base as shown by a shift of the absorption maximum, its photocycle contains a step with a chloride dependent rate, and it transports chloride in the cytoplasmic direction (Sasaki et al., 1995). Conversely, when the missing protonatable side chain of residue 85 is replaced by an azide anion bound to halorhodopsin at the site where normally chloride would bind, its Schiff base deprotonates upon photoisomerization of the retinal like in bacteriorhodopsin, and it transports protons in the extracellular direction (Váró et al., 1996). Halorhodopsin transports protons in the cytoplasmic direction when a protonation/deprotonation cycle of its Schiff base is driven by a 2-photon reaction (Bamberg et al., 1993).

Although these results indicate that the conduction pathways between the two membrane surfaces and the buried Schiff base−counter-ion complex are much less ion-specific than previously imagined, there may be segments in these pathways that are uniquely suited to transfer either protons or chloride. For example, if there is a structure along the pathway that exists explicitly for proton transfer, its presence should make itself felt in the binding and transport of chloride by D85T bacteriorhodopsin. The linkage of asp-85 and glu-204, by what seems to be an about 10 Å long hydrogen-bonded chain of bound water in the extracellular region of

† This work was funded partly by grants from the the National Institutes of Health (GM 29498 to J.K.L.), the Department of Energy (DEFG03-86ER13525 to J.K.L. and DEFG02-92ER20089 to R.N.), the National Science Foundation (MCB-9202209 to R.N.), and the U.S. Army Research Office (DAAL03-92-G-0406 to R.N.).

* Author to whom correspondence should be addressed.

‡ University of California.

§ Wayne State University.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: site-directed mutants are denoted with the residues in the wild type and mutant, separated by the residue number, *e.g.*, D85T or D85T/E204Q; BR, bacteriorhodopsin with *all-trans* chromophore; M and O, two of the intermediates of the BR photocycle; Bis-tris-propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid.

S0006-2960(96)02293-3 CCC: $12.00

the protein involving also arg-82 (Humphrey et al., 1994; Scharnagl et al., 1995), may be such a structure. It was recently shown that the pK_a's of these residues are so coupled to one another that when one becomes protonated near neutral pH the other dissociates, and *vice versa* (Balashov et al., 1995, 1996; Richter et al., 1996). During the proton transport cycle this linkage causes (a) the release of a proton from the extracellular surface from glu-204 (and perhaps bound water) upon protonation of asp-85 by the Schiff base and (b) the rise of the pK_a of asp-85 once the proton is released, ensuring the irreversibility of the loss of the Schiff base proton. In halorhodopsin the residue equivalent to glu-204 is a threonine and not protonatable, but in D85T bacteriorhodopsin protonation and deprotonation of glu-204 might be expected to affect the binding of chloride near thr-85. Inasmuch as the reverse should be also true and the pK_a of glu-204 is changed by the binding of chloride, protonation and deprotonation of glu-204 should correlate with the appearance and departure of chloride from the vicinity of the Schiff base during its transport.

We report here experiments with the D85T, D85S, and D85T/E204Q mutants of bacteriorhodopsin that explore the possibility of such a relationship between the vicinity of the Schiff base and the extracellular surface in the chloride-transporting bacteriorhodopsin mutants. As expected, the pK_a of a residue that we identify as glu-204 responds to the binding of chloride near the Schiff base, and this has two consequences: (a) glu-204 becomes protonated when the chloride binds and deprotonated when the chloride dissociates, and (b) the binding of chloride is pH dependent because of the protonation of glu-204. These observations made it possible to follow the presence or absence of chloride near the Schiff base through proton uptake or release from glu-204, and to establish that the red-shifted intermediate in the chloride transport cycle occurs after internal transfer of the bound chloride, presumably on the way to its release at the cytoplasmic surface but before its replacement by another chloride ion, presumably from the extracellular surface.

MATERIALS AND METHODS

The D85T, D85T/E204Q, D85T/D96A, and D85S mutants were constructed as described before (Needleman et al., 1991), and the proteins were prepared after homologous expression in *Halobacterium salinarium* as purple membranes by a standard method (Oesterhelt & Stoeckenius, 1974).

Spectra were measured with a Shimadzu UV-250 or a UV-1601 spectrophotometer. The spectra of the blue, purple, and yellow states (cf. below) were calculated by assuming that they are in equilibrium (according to the models discussed in the text) and extrapolating the spectra near the end-points to 100% pure states. In this way, pH titrations in the absence of chloride gave the spectra of the blue and yellow states. Inasmuch as the same states arose in both pH and chloride ion titrations, these were then used to calculate the spectra of the purple state. The spectra were then used to fit the spectral mixtures in order to calculate the relative contribution of each state. Transient absorption change was measured after photoexcitation with a 532 nm Nd:YAG laser pulse, as described before [*e.g.*, Váró et al. (1995a)]. Release and uptake of protons was measured with fluorescein covalently linked to lys-129 at the extracellular surface (with and without buffer), and with pyranine that is located in the bulk (with and without pyranine), as described by Heberle and Dencher (1992).

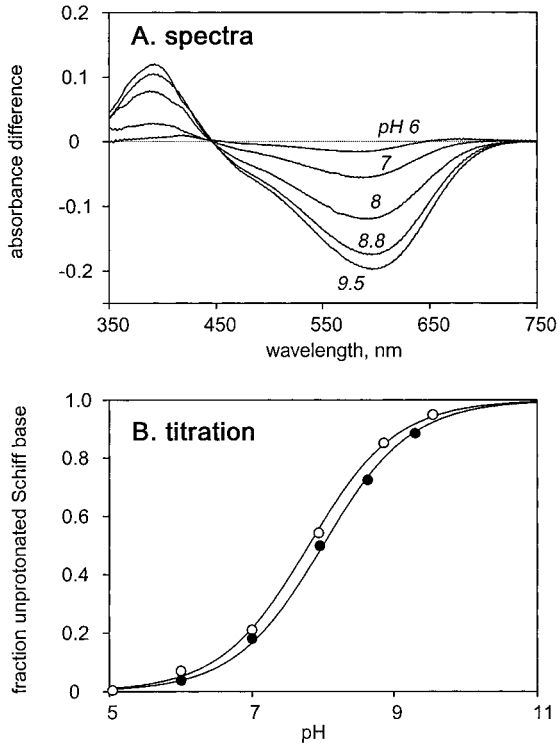

FIGURE 1: Titration of the Schiff base in D85T bacteriorhodopsin. A. Difference between spectra measured at pH 5.0 and the indicated pH, in 1 M Na_2SO_4. Bacteriorhodopsin at 4 mM. B. Fraction of unprotonated Schiff base, calculated from difference spectra as in A, as a function of pH, in either 1 M Na_2SO_4 (open circles) or 2 M NaCl (closed circles). In Na_2SO_4 the pK_a is 7.8 ($n = 0.7$); in NaCl the pK_a is 8.0 ($n = 0.7$).

In all measurements, mixtures of 1 M Na_2SO_4 and 2 M NaCl were used to keep the [Na^+] constant while the [Cl^-] was varied. Buffering was with 25 mM each of Bis-tris-propane, CAPS, succinate, and phosphate in the titrations of the Schiff base and with 25 mM each of succinate and phosphate in the titrations with chloride.

RESULTS

pK_a of the Protonated Schiff Base in D85T, D85S, and D85T/E204Q Bacteriorhodopsins. When asp-85 is replaced with a neutral residue the Schiff base pK_a is lowered by 4−5 pH units (Otto et al., 1990; Thorgeirsson et al., 1991; Turner et al., 1993; Brown et al., 1993; Tittor et al., 1994), and this is true also for D85T (Brown et al., 1993; Tittor et al., 1994). Figure 1A shows difference spectra between pH 5 and various pH values that indicate the disappearance of the chromophore with protonated Schiff base at 598 nm (blue state) and the appearance of the unprotonated Schiff base at 392 nm (yellow state). The negative absorbance band has a greater half-width than usual because this mutant contains a considerable amount of 13-*cis* chromophore[2] with a maximum somewhat blue-shifted from the *all-trans*. In 1 M Na_2SO_4 the pK_a of the Schiff base D85T is 7.8. In 2 M NaCl it is 8.0 (Figure 1B), *i.e.*, the pK_a is virtually unaffected by chloride. D85S behaved similarly. The pK_a in sulfate was 8.2, and in chloride it was 8.5 (not shown).

[2] Determined from the fingerprint region of FT-Raman spectra (Brown and Lanyi, unpublished experiments).

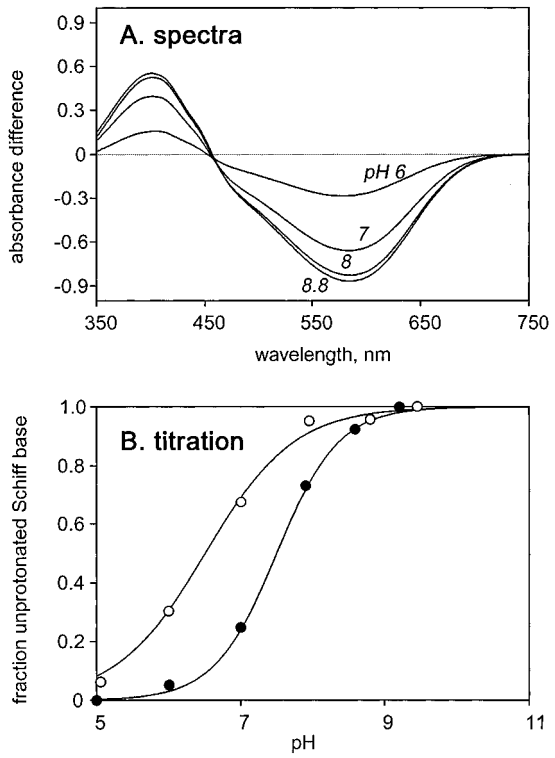

FIGURE 2: Titration of the Schiff base in D85T/E204Q bacteriorhodopsin. A. Difference between spectra measured at pH 5.0 and the indicated pH, in 1 M Na_2SO_4. Bacteriorhodopsin at 14 mM. B. Fraction of unprotonated Schiff base, calculated from difference spectra as in A, as a function of pH, in either 1 M Na_2SO_4 (open circles) or 2 M NaCl (closed circles). In Na_2SO_4 the pK_a is 6.5 (n = 0.7); in NaCl the pK_a is 7.5 (n = 1.0).

In D85T/E204Q this titration gives a somewhat different result. The negative band in sulfate is shifted to 586 nm (Figure 2A). The shift originates directly from the additional E204Q residue change because the isomeric composition of the retinal is not significantly affected.[2] More importantly, the pK_a of the protonated Schiff base is lower in D85T/E204Q than in D85T and is significantly affected by chloride, *i.e.*, it is 6.5 in 1 M Na_2SO_4 and 7.5 in 2 M NaCl (Figure 2B).

Chloride-Dependent Spectral Changes in D85T and D85S Bacteriorhodopsins. Although chloride does not affect the deprotonation of the Schiff base in D85T, it causes a shift from the blue state to a purple one, as expected if the bound chloride contributes to the negative charge of the Schiff base counter-ion. Figure 3A shows difference spectra at pH 6 that illustrate the blue-to-purple shift that consist of a negative and a positive difference band at 627 and 534 nm, respectively. The absorption maximum of the pure purple state is at 554 nm. A small negative feature appears near 400 nm, due partly to change in the 13-*cis*/*all-trans* ratio and partly to some protonation of the Schiff base.

The amounts of the blue and purple states of D85T (as well as the small amount of yellow state at pH 6 that absorbs near 400 nm), are shown as functions of chloride concentration at pH 5 and 6 in Figure 4. The apparent dissociation constant for chloride is distinctly higher at pH 6 than at 5 (panels B and A, respectively). At pH 7 the dissociation constant was so high that little of the purple species could be seen at even 2 M chloride (not shown). If the small amount of chloride-independent yellow state is ignored, the blue/purple equilibrium can be modeled with a simple kinetic

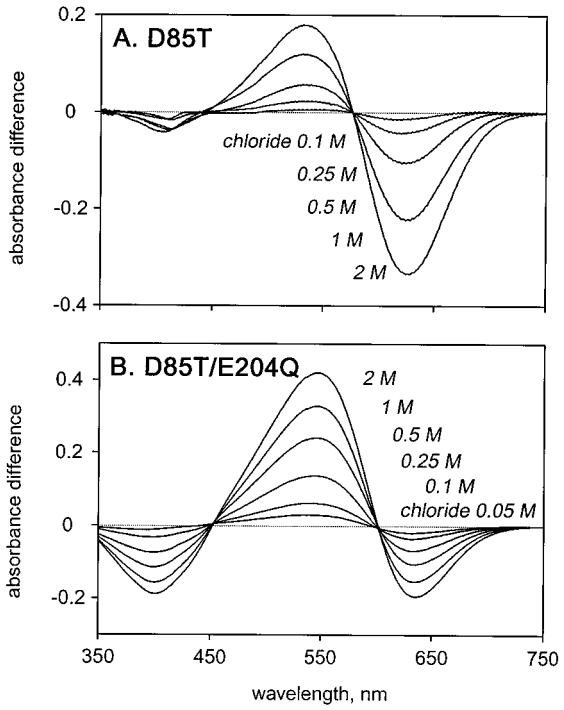

FIGURE 3: Chloride-dependent difference spectra at pH 6.0 for D85T (A) and D85T/E204Q (B) bacteriorhodopsins. The differences between spectra measured in 1 M Na_2SO_4 and in mixtures of 1 M Na_2SO_4 and 2 M NaCl to give the indicated chloride concentrations are shown. Bacteriorhodopsin at 12 and 16 mM in A and B, respectively.

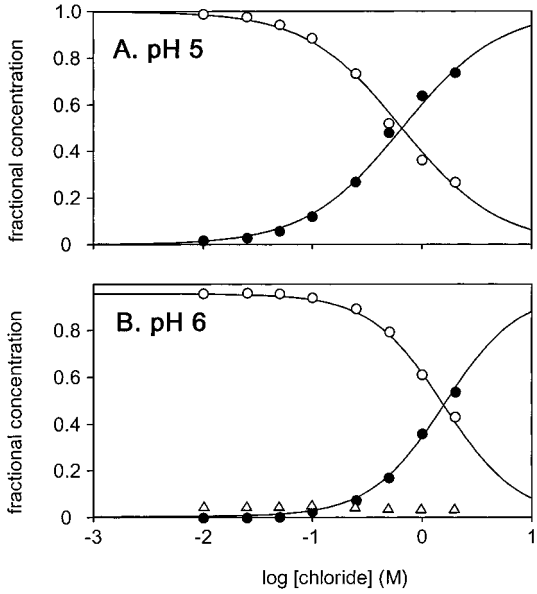

FIGURE 4: Spectroscopic titration of D85T bacteriorhodopsin with chloride. The blue state (protonated Schiff base, no bound chloride, open circles), the purple state (protonated Schiff base, bound chloride, closed circles), and the yellow state (unprotonated Schiff base, no bound chloride, triangles) are shown as functions of chloride concentration at pH 5 (A) and 6 (B). Solid lines: nonlinear least-squares best fit of eq 1 to the data. The calculated dissociation constant for chloride is 490 mM, and the pK_a of glu-204 is 5.4.

scheme (the lines in Figure 4), as follows:

$$\mathrm{BR{\cdot}Glu204^- + H^+ \Leftrightarrow}$$
$$\mathrm{BR{\cdot}Glu204H + Cl^- \Leftrightarrow BR{\cdot}Glu204H{\cdot}Cl^-} \quad (1)$$

where the protonation of glu-204 is described by a pK_a, and the binding of chloride to the protein with protonated glu-

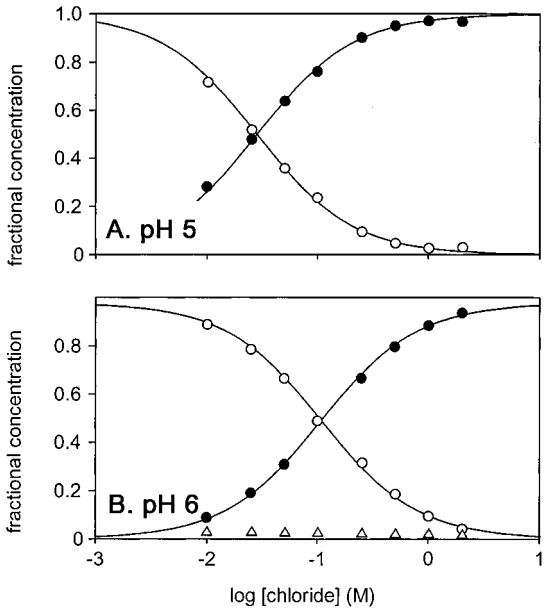

FIGURE 5: Spectroscopic titration of D85S bacteriorhodopsin with chloride. The blue state (protonated Schiff base, no bound chloride, open circles), the purple state (protonated Schiff base, bound chloride, closed circles), and the yellow state (unprotonated Schiff base, no bound chloride, triangles) are shown as functions of chloride concentration at pH 5 (A) and 6 (B). Solid lines: nonlinear least-squares best fit of eq 1 to the data. The calculated dissociation constant for chloride is 19 mM, and the pK_a of glu-204 is 5.3.

204 is described by a dissociation constant. We assume in this model that the pH dependence of the chloride binding is caused by the fact that the chloride will bind only if a residue becomes protonated. This residue is obviously not the retinal Schiff base, because (a) its pK_a would be then raised by chloride and this is not the case (Figure 1B), and (b) it is almost completely protonated throughout (Figures 3A and 4). If the analogy of the deprotonation of asp-85 in the wild type and the binding of the chloride anion near thr-85 in D85T is valid, then the linkage that exists between residues 85 and 204 (Balashov et al., 1995, 1996; Richter et al., 1996) predicts that the protonating residue should be glu-204. This scheme fits the data points in Figure 4 well if the dissociation constant for chloride (with glu-204 protonated) is 490 mM and the pK_a for glu-204 (without bound chloride) is 5.4. The former is reasonable since in D85T/E204Q, where residue 204 is uncharged, the binding constant for chloride is similar, *i.e.*, 360 mM (cf. below). The latter is also reasonable since the pK_a for glu-204 in the wild type, with asp-85 protonated, is 4.7−4.8 (Balashov et al., 1995; Richter et al., 1996).

Figure 5 shows the same kind of data, and the fit of the scheme, for D85S. The model fits the titration data with a dissociation constant of 19 mM for chloride and a pK_a of 5.3 for glu-204. Importantly, the affinity for chloride is greatly increased when residue 85 is a serine rather than a threonine and confirms the idea that the chloride binds in or near the space left upon replacing the aspartate with hydrogen-bonding side chains of smaller volume. Conversely, no chloride-dependent spectral shifts are observed in D85N in this pH region (Chon et al., 1996), as expected from the requirement for creating a suitable cavity for the chloride binding. Experiments not shown here indicated that cell envelope vesicles containing D85S, but not D85N,

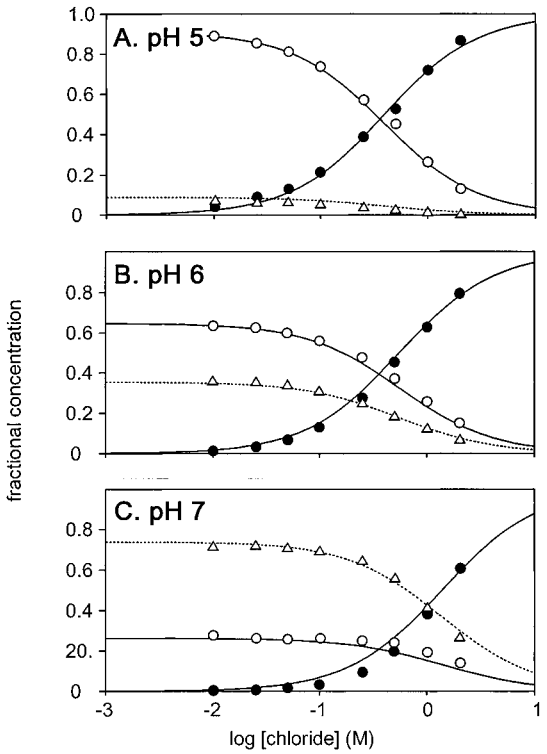

FIGURE 6: Spectroscopic titration of D85T/E204Q bacteriorhodopsin with chloride. The blue state (protonated Schiff base, no bound chloride, open circles), the purple state (protonated Schiff base, bound chloride, closed circles), and the yellow state (unprotonated Schiff base, no bound chloride, triangles) are shown as functions of chloride concentration at pH 5 (A), 6 (B), and 7 (C). Solid lines: nonlinear least-squares best fits of eq 2 to the data. The dissociation constant for chloride is 360 mM, and the pK_a of the retinal Schiff base is 6.4.

bacteriorhodopsin exhibited light-dependent chloride transport like D85T.

Chloride-Dependent Spectral Changes in D85T/E204Q Bacteriorhodopsin. Residue 204 is unavailable for protonation or deprotonation in the double mutant D85T/E204Q, and its chloride-dependent spectral properties are indeed different. Figure 3B shows that, consistent with the lower pK_a of the Schiff base in this mutant and its dependence on chloride (Figure 2B), addition of chloride at pH 6 causes absorption decreases at both 400 and 637 nm and an increase at 549 nm, *i.e.*, adding chloride converts the mixture of yellow and blue species in sulfate to the purple species.

In the titration of D85T/E204Q with chloride at pH 5, 6, and 7 (Figure 6) the blue and yellow states are converted to the purple state with a single apparent dissociation constant for chloride that increases with pH. The lines in Figure 6 are the best fits of the scheme in eq 2

$$BR{\cdot}SB + H^+ \Leftrightarrow BR{\cdot}SBH^+ + Cl^- \Leftrightarrow BR{\cdot}SBH^+{\cdot}Cl^- \quad (2)$$

where the residue that must protonate before chloride can bind is naturally not residue 204 which is now a glutamine, but the Schiff base which is designated as SB when unprotonated and SBH^+ when protonated. This interpretation is strictly required by the fact that in Figure 6 the blue−purple−yellow color changes completely define both the occupancy of the chloride binding site and the protonation state of the Schiff base. The best fit of the model to the chloride-dependent concentrations of the three states at the three pH values tested (lines in Figure 6) gives a pK_a for the Schiff

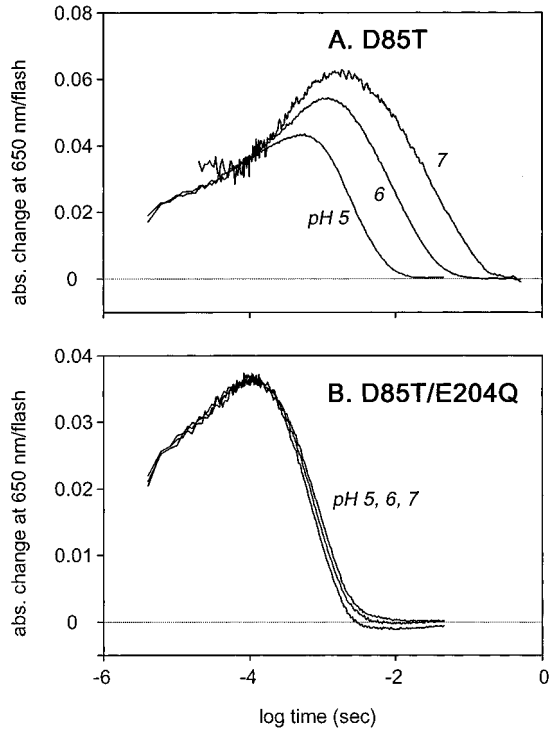

FIGURE 7: Transient absorbance change at 650 nm after photoexcitation of D85T (A) and D85T/E204Q (B) in 2 M NaCl at pH 5, 6, and 7. The amplitudes vary with pH because the red-shifted O intermediate originates from the chloride-bound forms (Sasaki et al., 1995), and the concentrations of these are pH dependent. For the comparison the traces were normalized at time regions that precede the O state. Bacteriorhodopsin at 12 and 14 mM in A and B, respectively.

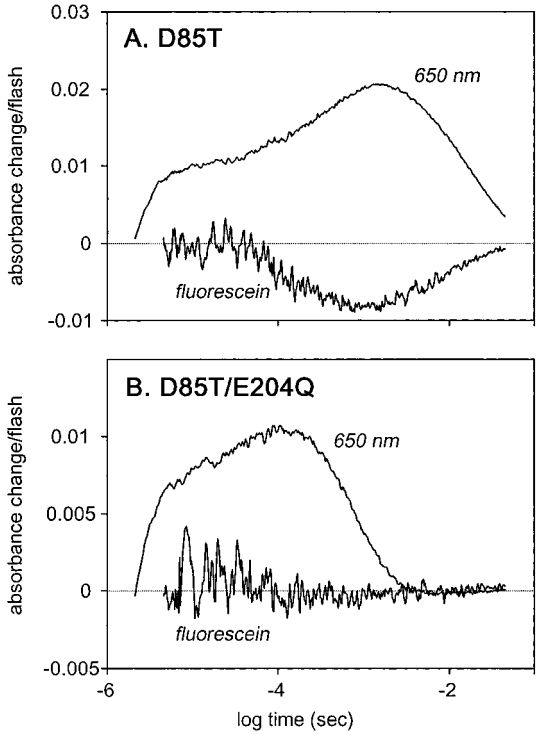

FIGURE 8: Proton release measured with covalently bound fluorescein in D85T (A) and D85T/E204Q (B). Conditions: 2 M NaCl, pH 6.1, bacteriorhodopsin, labeled with carboxyfluorescein succinimidyl ester at lys-129 (Heberle & Dencher, 1992), at 4 mM in both A and B. Traces labeled with *650 nm* are absorbance changes at this wavelength. Traces labeled with *fluorescein* were measured at 490 nm, and a second trace measured at the same pH but with 40 mM phosphate was subtracted.

base as 6.4, in good agreement with its value of 6.5 from direct titration in the absence of chloride (Figure 2B). The calculated dissociation constant for chloride is 360 mM. It appears, therefore, that while the pH dependence of chloride binding in D85T originates from the protonation of glu-204 with virtually no involvement of protonation of the Schiff base, the pH dependence in D85T/E204Q originates entirely from protonation of the Schiff base.

Protonation Changes in the Photocycles of D85T, D85T/E204Q, and D85T/D96A. Figure 7A shows absorption change with time at 650 nm after photoexcitation of D85T. The early phase, up to tens of microseconds, is the absorbance of the K intermediate [seen in both bacteriorhodopsin and halorhodopsin, cf. Zimányi et al. (1989)], and this is followed by a late red-shifted state. The decay of this photointermediate is seen to be not only chloride dependent (Sasaki et al., 1995) but also pH dependent, and is slower with increasing pH. The residue that confers this pH dependence is glu-204 because no such pH effect is seen in D85T/E204Q (Figure 7B).

This pH dependence in D85T is as expected from the earlier suggested chloride translocation mechanism. According to eq 1, if the chloride moves away from its binding site during the rise of the red-shifted intermediate (Váró et al., 1995a,b), the protonated glu-204 should lose its proton (depending on the pH and its effective pK_a at this time). Conversely, when chloride is bound again during the decay of this intermediate, completing the chloride transfer pathway, the anionic glu-204 must become protonated. Thus, we expect that in D85T, but not in D85T/E204Q, protons will be released together with the rise of the red-shifted intermediate, and protons will be taken up together with its decay. Figure 8 shows results with the pH indicator dye fluorescein covalently bound to the lys-129 at the extracellular surface (Heberle & Dencher, 1992), that indicate that this is indeed the case. Similar results were obtained with pyranine (Heberle & Dencher, 1992), a dye that detects pH changes in the bulk (not shown). In the absence of chloride neither the red-shifted photointermediate of D85T nor the proton release was observed (measurement in 1 M Na_2SO_4, not shown).

The proton release is absent in D85T/E204Q even in 2 M NaCl (Figure 8B), and this strongly implicates glu-204 as the reversibly protonated residue. Another control experiment was with D85T/D96A, which had a photocycle like D85T and released protons like D85T (not shown). Asp-96, the other protonated residue in the protein, is therefore not the origin of the proton detected, and the cytoplasmic region where it is located is not involved in these proton transfer reactions. Thus, consistent with lack of proton transport in D85T under these conditions (Sasaki et al., 1995), the release and uptake seen must both occur at the extracellular surface where glu-204 is located. The proton release is not required for chloride transport because D85T/E204Q transports chloride like D85T (not shown).

DISCUSSION

Little is known at this time about the molecular details of the binding and the translocation of chloride in halorhodopsin [for a recent review, see Oesterhelt (1995)]. The various carboxyl groups that span the width of bacteriorhodopsin

and act as proton donors and acceptors have been identified by infrared spectroscopy [for a recent review, see Maeda (1995)], but there is only limited information on the binding and transfer of chloride from site to site during its transport in halorhodopsin or D85T bacteriorhodopsin (Walter & Braiman, 1994; Rüdiger et al., 1995; Chon et al., 1996). The measurements we report here include the absorption maximum of the retinal chromophore and the protonation state of groups that may be influenced by the presence of bound chloride, as well as the pH dependence that the proton binding confers onto the binding of the chloride.

According to titration of unphotolyzed halorhodopsin with respect to both chloride and pH, chloride is bound only when the Schiff base is protonated (Váró et al., 1996). The D85T/E204Q bacteriorhodopsin mutant behaves very similarly (Figure 6). This is understandable since the two protonatable residues that appear to influence the Schiff base have been replaced so as to make this protein resemble halorhodopsin. Thus, the chloride ion and pH-dependent equilibrium of the blue, purple, and yellow states (with protonated Schiff base but no bound chloride, protonated Schiff base with bound chloride, and unprotonated Schiff base, respectively) is described by the halorhodopsin-like model of eq 2. As in halorhodopsin, the chloride binding site in this bacteriorhodopsin mutant must be near residue 85 because D85S binds the anion with much greater affinity (Figure 5), but it is also near the protonated Schiff base because its occupancy is dependent on its positive charge (Figure 6). Conversely, as in halorhodopsin the additional negative charge of the bound chloride ion in D85T/E204Q bacteriorhodopsin stabilizes the protonated Schiff base and thereby raises its pK_a.

However, the bacteriorhodopsin mutant D85T, where residue 204 is a glutamate, exhibits different behavior, with features characteristic of both bacteriorhodopsin and halorhodopsin. Unlike in D85T/E204Q, in D85T the Schiff base pK_a is now high enough to keep it protonated in the entire pH region where chloride binds. The reason is presumably the long-distance influence of glu-204 that is anionic already at the pH where the Schiff base begins to deprotonate (the two pK_a of these groups being 5.4 and 7.8, respectively). That the anionic glu-204 has a direct influence on the protonated Schiff base is suggested also by a difference in the absorption maxima of D85T and D85T/E204Q in sulfate (Figures 1A and 2A). As a consequence of its high pK_a, the protonation state of the Schiff base can have no effect on chloride binding (Figure 4B). Indeed in D85T the Schiff base pK_a is unaffected by the presence of chloride (Figure 1B). The observed pH dependence for chloride binding must arise in another way. There is a coupling between the protonation states of asp-85 and glu-204 that serves the purpose of proton translocation in wild type bacteriorhodopsin (Balashov et al., 1995, 1996; Richter et al., 1996). If the appearance of a negative charge upon binding of chloride at thr-85 is equivalent to deprotonation of asp-85, then binding of chloride would require that glu-204 become protonated. In this case chloride and proton will both bind to D85T, the chloride near thr-85 and the proton to glu-204, and this would confer a pH dependence onto the chloride binding. Such a mechanism, described by eq 1, fits the data. The apparent affinity for chloride increases with decreasing pH in the manner required (Figure 4), and although it has not been measured spectroscopically like the protonation state of the Schiff base, this implies that the pK_a of glu-204 is raised in turn with increasing chloride concentration.

According to this mechanism, the protonation state of glu-204 will report on the charge environment of the Schiff base, and in D85T in particular on the presence of chloride in this location. We used this to examine the chloride switch in the chloride transport cycle. In the proton transport mechanism for bacteriorhodopsin the M intermediate plays the key role because it represents the Schiff base after its proton is lost toward the extracellular side but before its reprotonation from the cytoplasmic side. The proton switch, *i.e.*, the change of connectivity from one side to the other, occurs during the lifetime of this state (Henderson et al., 1990; Váró & Lanyi, 1991; Kataoka et al., 1994). What intermediate plays this role during the chloride transport? In the cycle of halorhodopsin (Scharf & Engelhard, 1994; Váró et al., 1995a,b), as well as D85T (Sasaki et al., 1995), there is a red-shifted intermediate that arises on the millisecond time scale. In the case of *Natronobacterium pharaonis* halorhodopsin at least, its infrared spectrum indicates that it occurs after reisomerization because unlike in the preceding state, which contains 13-*cis* retinal, it has *all-trans* retinal (Váró et al., 1995a) like the O state at the end of the bacteriorhodopsin photocycle (Smith et al., 1983). Because the observed red-shift could originate from the absence of chloride near the Schiff base that would make the counterion less negative, we had suggested that this state might play the same role as M in the bacteriorhodopsin photocycle (Váró et al., 1995b). If so, the rise of this intermediate reflects the transfer of the sequestered chloride from the vicinity of the Schiff base to the cytoplasmic region, where it is eventually released to the surface. Its decay reflects, in turn, the rebinding of chloride. Decay of the red-shifted O-like intermediate is indeed faster with increasing chloride concentration in both *N. pharaonis* halorhodopsin (Váró et al., 1995b) and D85T (Sasaki et al., 1995), suggesting that the transported chloride is replaced upon recovery of the initial state, presumably from the extracellular side. If this is so, we should expect that the pK_a of glu-204 in the D85T mutant will first become lower and then higher as the bound chloride leaves and then replaced, and therefore a proton should be released and taken up from the bulk at the time of the rise and the decay of the red-shifted photointermediate, respectively. This is indeed observed (Figure 8A), and the absence of the protonation changes in the D85T/E204Q mutant (Figure 8B) is consistent with glu-204 as the the origin of the released proton. The results thus strongly support the suggested mechanism.

For comparing the chloride and proton transport mechanisms, we will describe them in the most general terms possible, as follows. In the proton transport cycle of bacteriorhodopsin the proton switch occurs in the extracellular to cytoplasmic direction (*i.e.*, in the opposite direction from the transport) and during the M state (*i.e.*, while the proton binding site, which is the Schiff base, is unoccupied). The resetting of the switch has not been investigated in detail but must occur in the cytoplasmic to extracellular direction and after the N intermediate (*i.e.*, while the proton binding site is occupied). In the chloride transport cycle the proposed chloride switch during the O state is in the cytoplasmic to extracellular direction (*i.e.*, in the opposite direction from the transport) and with the chloride binding site unoccupied.

This is entirely analogous to the proton switch in the M intermediate, except that in M the retinal is 13-*cis* while in O it is *all-trans*. Whether the resetting of the chloride switch occurs before or after the O state depends on the direction of the initial accessibility of the chloride binding site. The direction of this accessibility is not yet clear. To explain the direction of proton transport in two-photon experiments with halorhodopsin and D85X bacteriorhodopsins, Oesterhelt and co-workers suggested that the initial access of the Schiff base is from the extracellular side (Bamberg et al., 1993; Tittor et al., 1994). In this case, the resetting of the switch, of necessity in the extracellular to cytoplasmic direction and with the binding site occupied, must occur earlier in the photocycle than the O state (Oesterhelt, 1995). On the other hand, if the accessibility in unphotolyzed D85X bacteriorhodopsins is from the cytoplasmic side, as suggested by the influence of the side chain of residue 96 and the presence of azide on the pH jump-induced deprotonation of the Schiff base in D85N/D96X mutants (Kataoka et al., 1994), the resetting of the switch must occur after the O state.

REFERENCES

Balashov, S. P., Govindjee, R., Imasheva, E. S., Misra, S., Ebrey, T. G., Feng, Y., Crouch, R. K., & Menick, D. R. (1995) *Biochemistry 34*, 8820−8834.

Balashov, S. P., Imasheva, E. S., Govindjee, R., & Ebrey, T. G. (1996) *Biophys. J. 70*, 473−481.

Bamberg, E., Tittor, J., & Oesterhelt, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 639−643.

Braiman, M. S., Walter, T. J., & Briercheck, D. M. (1994) *Biochemistry 33*, 1629−1635.

Brown, L. S., Bonet, L., Needleman, R., & Lanyi, J. K. (1993) *Biophys. J. 65*, 124−130.

Chon, Y.-S., Sasaki, J., Kandori, H., Brown, L. S., Lanyi, J. K., Needleman, R., & Maeda, A. (1996) *Biochemistry 35*, 14244−14250.

Ebrey, T. G. (1993) in *Thermodynamics of Membranes, Receptors and Channels* (Jackson, M., Ed.) pp 353−387, CRC Press, New York.

Heberle, J., & Dencher, N. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5996−6000.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899−929.

Humphrey, W., Logunov, I., Schulten, K., & Sheves, M. (1994) *Biochemistry 33*, 3668−3678.

Kataoka, M., Kamikubo, H., Tokunaga, F., Brown, L. S., Yamazaki, Y., Maeda, A., Sheves, M., Needleman, R., & Lanyi, J. K. (1994) *J. Mol. Biol. 243*, 621−638.

Lanyi, J. K. (1990) *Physiol. Rev. 70*, 319−330.

Lanyi, J. K. (1993) *Biochim. Biophys. Acta 1183*, 241−261.

Maeda, A. (1995) *Isr. J. Chem. 35*, 387−400.

Mathies, R. A., Lin, S. W., Ames, J. B., & Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 491−518.

Needleman, R., Chang, M., Ni, B., Váró, G., Fornes, J., White, S. H., & Lanyi, J. K. (1991) *J. Biol. Chem. 266*, 11478−11484.

Oesterhelt, D. (1995) *Isr. J. Chem. 35*, 475−494.

Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667−678.

Oesterhelt, D., Tittor, J., & Bamberg, E. (1992) *J. Bioenerg. Biomembr. 24*, 181−191.

Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018−1022.

Richter, H. T., Brown, L. S., Needleman, R., & Lanyi, J. K. (1996) *Biochemistry 35*, 4054−4062.

Rüdiger, M., Haupts, U., Gerwert, K., & Oesterhelt, D. (1995) *EMBO J. 14*, 1599−1606.

Sasaki, J., Brown, L. S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R., & Lanyi, J. K. (1995) *Science 269*, 73−75.

Scharf, B., & Engelhard, M. (1994) *Biochemistry 33*, 6387−6393.

Scharnagl, C., Hettenkofer, J., & Fischer, S. F. (1995) *J. Phys. Chem. 99*, 7787−7800.

Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. A. (1983) *Biochemistry 22*, 6141−6148.

Thorgeirsson, T. E., Milder, S. J., Miercke, L. J. W., Betlach, M. C., Shand, R. F., Stroud, R. M., & Kliger, D. S. (1991) *Biochemistry 30*, 9133−9142.

Tittor, J., Schweiger, U., Oesterhelt, D., & Bamberg, E. (1994) *Biophys. J. 67*, 1682−1690.

Turner, G. J., Miercke, L. J. W., Thorgeirsson, T. E., Kliger, D. S., Betlach, M. C., & Stroud, R. M. (1993) *Biochemistry 32*, 1332−1337.

Váró, G., & Lanyi, J. K. (1991) *Biochemistry 30*, 5016−5022.

Váró, G., Brown, L. S., Sasaki, J., Maeda, A., Needleman, R., & Lanyi, J. K. (1995a) *Biochemistry 34*, 14490−14499.

Váró, G., Needleman, R., & Lanyi, J. K. (1995b) *Biochemistry 34*, 14500−14507.

Váró, G., Brown, L. S., Needleman, R., & Lanyi, J. K. (1996) *Biochemistry 35*, 6604−6611.

Walter, T. J., & Braiman, M. S. (1994) *Biochemistry 33*, 1724−1733.

Zimányi, L., & Lanyi, J. K. (1989) *Biochemistry 28*, 1662−1666.

BI9622938