Proton Transport by Proteorhodopsin Requires that the Retinal Schiff Base Counterion Asp-97 Be Anionic†

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ABSTRACT: At pH >7, proteorhodopsin functions as an outward-directed proton pump in cell membranes, and Asp-97 and Glu-108, the homologues of the Asp-85 and Asp-96 in bacteriorhodopsin, are the proton acceptor and donor to the retinal Schiff base, respectively. It was reported, however [Friedrich, T. et al. (2002) *J. Mol. Biol.*, *321*, 821−838], that proteorhodopsin transports protons also at pH <7 where Asp-97 is protonated and in the direction reverse from that at higher pH. To explore the roles of Asp-97 and Glu-108 in the proposed pumping with variable vectoriality, we compared the photocycles of D97N and E108Q mutants, and the effects of azide on the photocycle of the E108Q mutant, at low and high pH. Unlike at high pH, at a pH low enough to protonate Asp-97 neither the mutations nor the effects of azide revealed evidence for the participation of the acidic residues in proton transfer, and as in the photocycle of the wild-type protein, no intermediate with unprotonated Schiff base accumulated. In view of these findings, and the doubts raised by absence of charge transfer after flash excitation at low pH, we revisited the question whether transport occurs at all under these conditions. In both oriented membrane fragments and liposomes reconstituted with proteorhodopsin, we found transport at high pH but not at low pH. Instead, proton transport activity followed the titration curve for Asp-97, with an apparent pK_a of 7.1, and became zero at the pH where Asp-97 is fully protonated.

Proteorhodopsin is a eubacterial retinal-protein with considerable amino acid sequence similarity to bacteriorhodopsin (*1*, *2*). As bacteriorhodopsin, it is a light-driven proton pump (*1*), and Asp-97 and Glu-108, the homologues of Asp-85 and Asp-96 in bacteriorhodopsin (*1*), function as proton acceptor and donor to the retinal Schiff base, respectively (*3*). An interesting difference from bacteriorhodopsin, however, is that in proteorhodopsin the pK_a of Asp-97 is near 7 (*3*−*5*), rather than about 2.5 as for its homologue in bacteriorhodopsin (*6*, *7*). Below pH 7, the protonated Asp-97 cannot function as counterion to the positively charged protonated Schiff base, and the absorption maximum of the chromophore is red-shifted (*3*, *4*). In this pH region, Asp-97 will not function as proton acceptor, and no intermediate analogous to M[1] in bacteriorhodopsin, with a deprotonated Schiff base, accumulates in the photocycle (*3*−*5*). All of these properties resemble those of bacteriorhodopsin below pH 2.5, and bacteriorhodopsin does not transport protons at such low pH (*8*).

Early reports (*1*) on light-induced pH-changes in suspension of vesicles and/or liposomes described outward[2] (i.e., bacteriorhodopsin-like) proton translocation at pH above the pK_a of Asp-97 (see also ref *3*). However, a later report proposed, from photocurrents measured with proteorhodopsin-containing liposomes adsorbed to planar lipid films, that proteorhodopsin transports protons at pH as low as 5.2 (*4*), (i.e., well below the pK_a of Asp-97) and that this transport is in the reverse direction from transport at high pH.

In bacteriorhodopsin in the absence of the normal proton acceptor, in the D85N mutant, single-photon transport was reported only upon illumination of the state with unprotonated Schiff base (*9*), an observation with ready conceptual (*10*) and mechanistic (*11*, *12*) explanations. Reversing the vectoriality of a pump simply by removing its primary proton acceptor is a more novel and challenging proposal. There are conceptual and experimental contradictions to be overcome. To explain the difference from bacteriorhodopsin, Friedrich et al. (*4*) suggested that there is an open proton conduction pathway in proteorhodopsin on the cytoplasmic side of the Schiff base, such as formed in bacteriorhodopsin only at a late stage of the photocycle. Thus, illumination at low pH would cause the Schiff base proton to be lost to the cytoplasmic side, while reprotonation

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[1] Abbreviations: K, L, M, N, intermediates of the photocycle; TCM, triple cysteine mutant, C107V/C156V/C175V; TRIS, tris(hydroxymethyl)aminomethane; MES, 2-[*N*-morpholino]ethanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

[2] The term outward is used to characterize the direction of proton movement in proteorhodopsin in the natural membrane of the cell (i.e., from cytoplasmic to extracellular.

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would be from the extracellular side. In this scenario, therefore, Asp-97 and Glu-108 do not have the same roles at low pH as at high pH and not the same roles played by their homologues in bacteriorhodopsin. Being protonated, Glu-108 could not be the proton acceptor to the protonated Schiff base (as Asp-85 in the bacteriorhodopsin photocycle), although it might be in the pathway of the release of this proton to the cytoplasmic surface. On the other hand, the protonated Asp-97 could be either the proton donor to the unprotonated Schiff base (as Asp-96 in the bacteriorhodopsin photocycle), or owing to its close vicinity it might be in the pathway of reprotonation from the extracellular side.

To explain the lack of an intermediate with unprotonated Schiff base (an M state) in the low pH photocycle, it was suggested (*4*) that an M state is produced but escapes detection because for kinetic reasons it does not accumulate. The same explanation would account for lack of accumulation of intermediate(s) in which acidic residues have changed protonation states (*4*). Another problem was that transport in the reverse direction was not observed at pH 5.5 when proteorhodopsin was expressed in *Xenopus laevis* oocytes (*4*). To explain this, transport by the low-pH form (inward) was suggested to be weak or inefficient as compared to the strong or efficient transport by the high-pH form (outward). For this reason, at pH 5.5 inward transport in the oocytes (although not in the proteorhodopsin-containing liposomes) is overwhelmed by outward transport from the few percent of the high-pH form present.

Thus, Friedrich et al. (*4*) proposed that proteorhodopsin is an extraordinary and unique pump, in which both transport directions are possible and determined by the presence or absence of a ready proton acceptor to the Schiff base (i.e., by whether Asp-97 is anionic or protonated). In view of the far-reaching implications of this suggestion, we explored possible mechanisms for such transport. We studied the effects of pH and azide on the photocycles of the D97N and E108Q mutants. The results do not confirm that a state with unprotonated Schiff base is absent only because it is kinetically hidden, although undetected formation of such a state cannot be entirely ruled out. However, the findings, as well as our recent observation that under flash illumination the movement of a full charge across the membrane was observed at pH 9.5 but not at pH 5 (*13*), led us to question the existence of proton transport at pH below the pK_a of the Asp-97. Our transport measurements, using pH and illumination conditions optimal for detecting the reported reverse transport (*4*), did not confirm the earlier observation that at low pH there is proton translocation with reverse vectoriality.

MATERIALS AND METHODS

Proteorhodopsin (6xHis-tagged at the C-terminus) was expressed in *Escherichia coli* as described before (*1*), and the pigment was generated by addition an excess of *all-trans* retinal to intact cells during induction. The D97N and E108Q mutants had been prepared by site-specific mutagenesis as described earlier (*3*). We constructed also a triple mutant, in which all three cysteines of the wild-type protein (Cys-107, Cys-156, and Cys-175 (*2*)) are replaced with valine (called TCM further), to avoid problems from oxidation of one or more of the cysteine residues upon storage. The latter causes changes in the photocycle and the pK_a of Asp-97 (our unpublished results). The properties of TCM are very similar to fresh wild-type protein but unlike the wild-type TCM proved to be free of changes from prolonged storage. Membranes from the D97N and E108Q mutants were used within a few days after preparation to avoid this problem.

His-tagged TCM-proteorhodopsin was solubilized in 1% *n*-octyl-β-D-glucopyranoside and purified on a Ni-NTA agarose column (Qiagen, Germany) to a ratio of protein to chromophore absorption bands, A_{280}/A_{530}, of ~2.2. Proteoliposomes were prepared by overnight dialysis of solubilized TCM proteorhodopsin plus lipid against 20 mM phosphate buffer at pH 8.5. Either the polar lipids extracted from purple membranes of *Halobacterium salinarum* as described in ref *14* or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, # 850375, Avanti Polar Lipids Inc., Alabama) were used for reconstitution, with either high (1:2) or low (1:20) protein-to-lipid ratios (w/w).

Flash photolysis and measurements of absorption changes in the visible range were performed as described earlier (*11*), with proteorhodopsin-containing membrane fragments encased in polyacrylamide gel (*3*), or proteoliposomes, at 23 °C. Light-induced pH-changes were recorded at 23 °C in 1−2 mL samples of proteoliposomes suspended in unbuffered solutions of either 20 or 100 mM NaCl during illumination with a 250W Tungsten−halogen lamp equipped with heat and cutoff filters (>500 nm). Under these illumination conditions (~43 mW/cm^2 in the 500−600 nm range, ~3 mW/cm^2 >600 nm), the expected rate of the light-induced reactions is ≤ 5 s^{-1} (i.e., $\tau \geq 200$ ms). The two slowest decay constants in the TCM photocycle are 10 ± 1 and 70 ± 20 ms at pH 9.5, or 11 ± 1 and 50 ± 20 ms at pH 5.0. Therefore, two-photon effects, because of re-excitation of slow intermediates, should be negligible.

A pH meter with a ROSS electrode (#8175, Orion Research Inc., Boston, MA) was used, whose output was digitized by an analogue-to-digital converter (Gage Compuscope 6012/PCI-4M, Gage Applied Inc., Lachine, Canada). For time-resolved photocycle measurements with proteoliposome suspensions, the pH was stabilized with 50 mM phosphate buffer.

Photoelectric currents under continuous illumination (light flux of ~50 mW/cm^2 in the wavelength range >500 nm) were recorded on membrane fragments oriented in electric field samples in 100 mM NaCl, 25 mM MES, 25 mM TRIS at 20 °C, as in ref *13*.

RESULTS AND DISCUSSION

Figures 1−3 show transient absorption changes at three characteristic wavelengths after flash illumination. The negative absorption changes at 500 nm originate from depletion of the initial state. The positive absorption changes at 590 nm indicate the accumulation of red-shifted intermediate(s). Any absorption change at 420 nm would indicate the accumulation of an M-like intermediate with unprotonated retinal Schiff base. M is present at high pH (Figures 2 and 3B) but is not seen at low pH (Figure 1), in accord with earlier reports (*3*, *4*, *13*). In the TCM mutant, used as a control (Figures 1A and 2A), the traces are very similar to the wild-type traces published earlier for low (*13*) and high (*3*, *4*, *15*) pH, respectively. At low pH (Figure 1) only red-

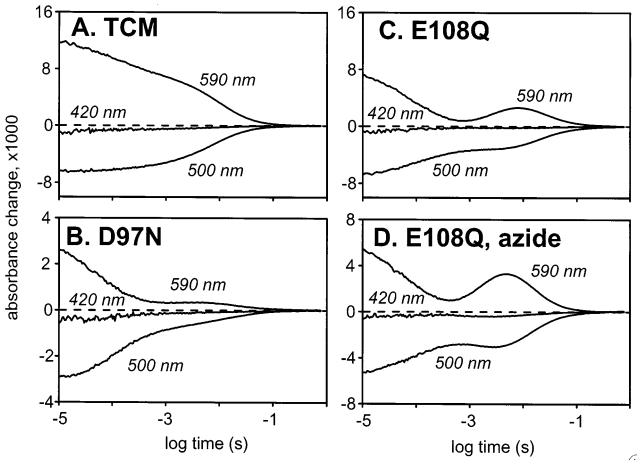

FIGURE 1: Flash-induced transient absorption changes in membrane fragments of pseudo-wild-type proteorhodopsin (triple cysteine mutant, TCM) and the mutants D97N and E108Q, encapsulated in polyacrylamide gel at pH 5.0 (50 mM succinate, 150 mM NaCl) at 23 °C. Absorption changes after a laser pulse were followed at 420, 500, and 590 nm, as indicated. (A) TCM; (B) D97N; (C) E108Q; and (D) E108Q with 100 mM NaN_3.

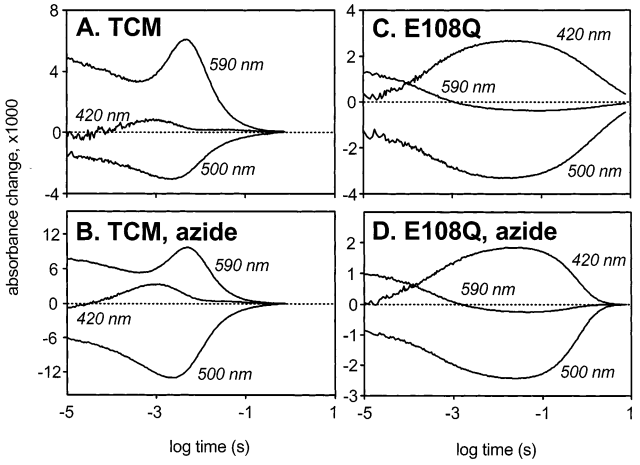

FIGURE 2: Flash-induced transient absorption changes in membrane fragments of pseudo-wild-type proteorhodopsin (triple cysteine mutant, TCM) and the mutants D97N and E108Q, encapsulated in polyacrylamide gel at pH 9.5 (50 mM CAPSO, 150 mM NaCl) at 23 °C. Absorption changes after a laser pulse were followed at 420, 500, and 590 nm, as indicated. (A) TCM; (B) TCM with 100 mM NaN_3; (C) E108Q; and (D) E108Q with 100 mM NaN_3.

shifted intermediates, a K- and an N-like[3] state (*3*), are apparent. At high pH (Figure 2), contributions from K-, M-, and red-shifted N-like intermediates are evident.

Photochemical Cycles of the D97N and E108Q Mutants of Proteorhodopsin. Time-resolved absorbance changes in the TCM, D97N, and E108Q mutants are presented in Figure 1A−C. The main feature is the absence of any detectable absorption change at 420 nm at low pH in any of these photocycles. At first sight, at the other two wavelengths the traces of D97N and E108Q appear very different from TCM, but this is not really the case. To a first approximation, all three photocycles are dominated by interconversions between

[3] As we have shown earlier (*3*), a late red-shifted intermediate, appearing on a millisecond time-scale in the PR photocycle, contains 13-*cis* retinal and therefore was called N to emphasize that this is the state before chromophore re-isomerization (i.e., before the O state (in BR), which is formed after re-isomerization).

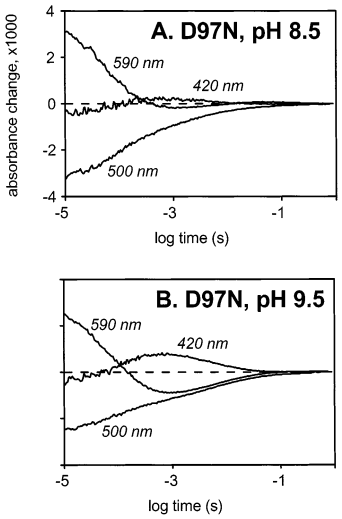

FIGURE 3: Flash-induced transient absorption changes in membrane fragments of the D97N mutant of proteorhodopsin encapsulated in polyacrylamide gel at pH 8.5 or 9.5 (50 mM CAPSO, 150 mM NaCl) at 23 °C. Absorption changes after a laser pulse were followed at 420, 500, and 590 nm, as indicated. (A) pH 8.5 and (B) pH 9.5.

several red-shifted states: K (or K substates) and later the red-shifted N (or N substates), as described in ref *13*, and it is the degree of their temporal overlap that defines the observable changes in the visible.

The main consequence of the D97N and E108Q mutations appears to be (Figure 1) that the late, red-shifted N intermediate accumulates to lesser extents than in the wild type and in the TCM samples, although the time-constants of its rise and decay are relatively unchanged (analysis not shown). The changed kinetics at 590 nm make the temporal separation of K and the red-shifted N state, and therefore, their separate and distinct existence, more evident. It is also clear from the traces in Figure 1B,C that there is an intermediate between K and the red-shifted N state, with an absorption spectrum less shifted than in either of these states. It might be the L intermediate, which was recently postulated from spectral analysis of the photocycle of the wild-type proteorhodopsin (*15*). Further, changed amplitudes but unchanged time-constants are consistent with equilibration (but not with unidirectional) reactions in the photocycle. Thus, the data for the mutants argue that equilibration reactions are essential to describe the proteorhodopsin photocycle. The high pH case was published earlier (*15*); the low pH photocycle is described separately (*13*).

From the similarity of the high-pH proteorhodopsin photocycle to the bacteriorhodopsin photocycle, it is expected that Glu-108 will be protonated at pH 5; therefore, in the low-pH photocycle it will not function as a proton acceptor. On the other hand, if the Schiff base proton is lost to the cytoplasmic side (as proposed in ref *4*), the E108Q mutation might be expected to affect its passage to the cytoplasmic surface, as was shown by comparing the photocycles of blue and yellow species in D85N and D85N/D96N mutants in bacteriorhodopsin (*11*, *16*). If the Schiff base deprotonation were made more rapid, an M state would accumulate. Conversely, if the Schiff base regains its proton from the extracellular side, the D97N mutation should affect this reaction. That is, if at low pH Asp-97 is the primary proton donor, the reprotonation of the Schiff base will be blocked

in D97N mutant. Alternatively, if Asp-97 is not the donor, its close proximity to the Schiff base will ensure that passage of the proton in the reprotonation reaction be strongly affected by replacement with Asn. If the reprotonation were made slower, again, an M state would accumulate. However, as shown in Figure 1B,C, there is no M state detected in either mutant. Thus, if a deprotonated Schiff base is produced in the photocycle, its deprotonation is not sufficiently rapid in E108Q, and its reprotonation is not sufficiently slow in D97N, to cause its accumulation. This evidence rules out direct participation of Asp-97 and Glu-108 in transport. Deprotonation of the Schiff base seems unlikely but not completely ruled out.

Effects of Azide on the Proteorhodopsin Photocycle. In our attempts to reveal the existence of an intermediate with deprotonated Schiff base, we took advantage of the fact that in the photocycle of D96N bacteriorhodopsin azide greatly accelerates proton transfer between the cytoplasmic surface and the retinal Schiff base. Azide is a weak acid, with a delocalized charge when anionic. HN_3 partitions into the hydrophobic cytoplasmic region and readily dissociates there. By providing a proton, it greatly accelerates the decay of the M state in the D96N mutant (*17*−*20*). Azide has much less effect on proton exchange on the extracellular side (i.e., on M rise in bacteriorhodopsin and its mutants (*11*, *16*, *21*, *22*)). In the proposed reverse transport in proteorhodopsin at low pH (*4*), it is the proton movement in the cytoplasmic region that affects the rise of the M state. Therefore, if azide has the same effects in proteorhodopsin as in bacteriorhodopsin, M rise should be accelerated, and this should lead to increase in the amount of observed M. At high pH, bacteriorhodopsin and proteorhodopsin pump in the same direction and employ the homologues Asp-85/Asp-97 and Asp-96/Glu-108 as primary proton acceptors and donors (*2*, *3*). Therefore, similarity of the azide-induced effect on the photocycle of proteorhodopsin at high pH will be direct evidence for the side-specificity of azide in this protein.

The effect of azide on proteorhodopsin at pH 9.5 is entirely as in bacteriorhodopsin. In Figure 2, kinetic traces are given for TCM and the E108Q mutant at pH 9.5. As in bacteriorhodopsin, azide has little effect on the kinetics of TCM proteorhodopsin (Figure 2A,B), but M decay is strongly accelerated in the E108Q mutant (Figure 2C,D), as in the D96N mutant of bacteriorhodopsin (*17*−*20*). In the E108Q photocycle at high pH, azide carries a proton from the cytoplasmic surface to the Schiff base and shuttles back as an anion; the active species being HN_3. Even at pH 9.5 where azide exists mostly as N_3^-, 100 mM azide caused an approximately 5-fold increase in the decay rate of M (Figure 2C,D). Azide substitutes for the missing internal proton donor, Glu-108, confirming that in proteorhodopsin, as in bacteriorhodopsin, the Schiff base is readily accessible to azide from the cytoplasmic side. As in bacteriorhodopsin, little effect was seen on M rise (Figure 2).

In D96N of bacteriorhodopsin, reprotonation of the Schiff base is strongly dependent on azide concentration and could be accelerated into the sub-ms range (*17*, *18*, *20*). At an azide concentration of 100 mM, at pH 9.5 the concentration of the active species, the proton donor HN_3, is about 1.6 μM (the pK_a of azide being 4.7 (*23*)). At pH 5.0, the concentration of the putative active species, the proton acceptor N_3^-, is far higher, at 66 mM. Thus, if the M state is produced at all

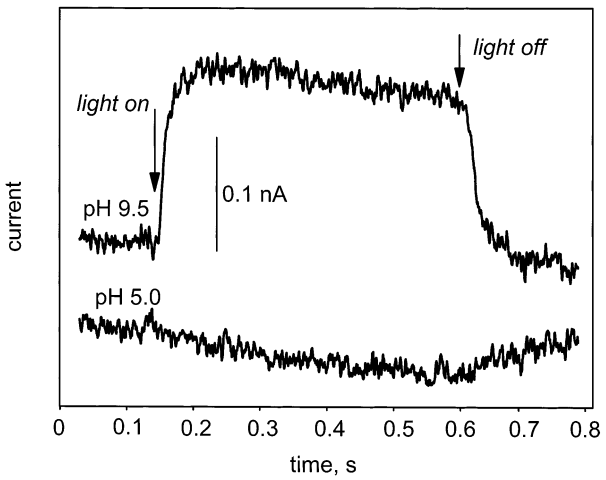

FIGURE 4: Photoelectric currents in membrane fragments of wild-type proteorhodopsin encapsulated in polyacrylamide gel under continuous illumination at >500 nm at pH 5.0 and 9.5 in 100 mM NaCl, 25 mM MES, 25 mM TRIS at 20 °C. The arrows indicate where the light was switched on and off.

in the E108Q mutant at pH 5.0, its formation should be strongly accelerated by 100 mM azide as was observed for the D85N/D96N mutant of bacteriorhodopsin (*16*). However, as shown in Figure 1C,D, no M state appears in the presence of 100 mM azide. The sole effect of azide appears to be an elevated accumulation of the red-shifted N state but with little change in its apparent rise and decay time-constants.

pH Dependency of the Photocycle of the D97N Mutant. In the D85N mutant of bacteriorhodopsin, little or no M intermediate is present in the photocycle at low pH, but M appears at pH >6 (*16*). There is no transport because deprotonation and reprotonation of the Schiff base both occur in the cytoplasmic direction (*16*). This demonstrated that in bacteriorhodopsin transport requires not only a lowering of the pK_a of the Schiff base in the photocycle but also that residue 85 function as a proton acceptor. D97N proteorhodopsin behaves in the same way. As shown in Figure 1B, no M is detected at pH 5. At pH 8.5, M accumulates to a small extent (Figure 3A), and at pH 9.5 (Figure 3B) the accumulation of M is about as high as in TCM. As the properties of the E108Q mutant resemble its bacteriorhodopsin homologue D96N, the D97N mutant closely resembles its bacteriorhodopsin homologue, D85N.

Thus, if there is proton transport at low pH in the direction opposite to that of the translocation at high pH, it will be based on an entirely different mechanism that does not utilize Asp-97 and Glu-108 as transient acceptor and donor. Nor are the acceptor and donor functions in this transport affected by replacement of Asp-97 and Glu-108 with Asn and Gln, respectively, even though in bacteriorhodopsin the homologous residues are at strategic locations in the proton-transfer pathways on the extracellular and cytoplasmic side of the Schiff base (*24*, *25*). To explore further the possibility of proton transport at low pH, we performed transport measurements in two different oriented systems.

Photoelectric Measurements Under Continuous Illumination. Membrane fragments containing proteorhodopsin exhibit electrical charge asymmetry and can be, therefore, oriented in an electric field (*15*). Figure 4 presents light-induced photoelectric signals in such oriented samples, with wild-type proteorhodopsin, under continuous illumination.

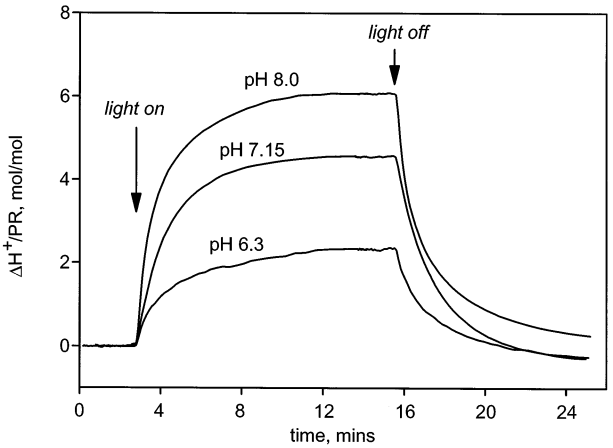

FIGURE 5: Time-course of light-induced acidification of unbuffered suspensions of proteorhodopsin (TCM)-containing liposomes (DOPC/proteorhodopsin ratio 20) in 20 mM NaCl under continuous illumination with light at >500 nm at pH 6.3, 7.15, and 8.0. The acidification (upward deflection) is shown as the number of protons pumped per proteorhodopsin (mol/mol).

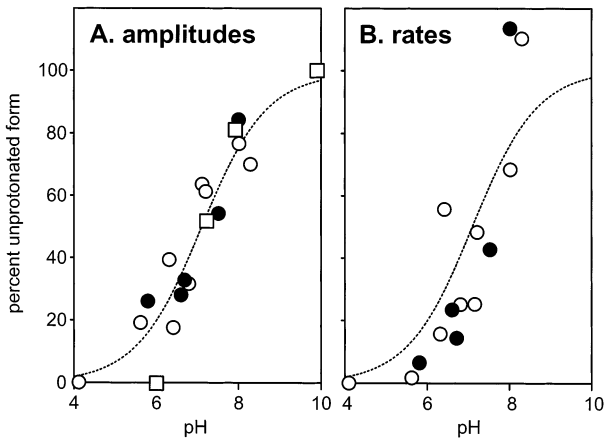

FIGURE 6: pH dependence of proton pumping in unbuffered suspensions of proteorhodopsin (TCM)-containing liposomes under continuous illumination at >500 nm. The points are from traces such as in Figure 5. Open circles, DOPC and closed circles, purple membrane lipids. For each batch of liposomes, 4−5 different pH-points were measured covering both low and high pH, and the data sets from the different preparations were scaled together. The dotted lines represent the best fits to all data points, with the absolute amplitudes of each data set for the different preparations treated as an adjustable parameter in fitting to the Henderson−Hasselbalch equation. For the latter, the parameters, $pK_a = 7.1$ and $n = 0.55$ were used, as obtained earlier from the spectroscopic titration of Asp-97 (*3*). (A) Steady-state displacement of protons from the liposomes during illumination. The 100% amplitude corresponds to about 10 protons/proteorhodopsin. Also shown are scaled values of transient absorption changes at 420 nm at several pH values (open rectangles) in DOPC liposomes. (B) Initial rates of proton transport. The 100% rate corresponds to about 0.2 mol protons/s per mol proteorhodopsin.

Earlier, in their interpretation of the light-intensity dependency of transport, Friedrich et al. (*4*) suggested that the outward transport at high pH might include contributions from both single- and two-photon processes, but the reversed transport at low pH is from a single-photon reaction. Therefore, although at higher light intensities the two-photon process seemed to dominate, at low intensities (below ~100 mW/cm^2) net reverse transport was observed, even at pH 7 (Figure 9 in ref *4*). Thus, the illumination level we used (~50 mW/cm^2) should have been low enough to produce the proposed reverse transport at or below pH 7. However, as shown in Figure 4, a steady-state photocurrent was observed at high (9.5) but not at low (5.0) pH. At pH 5.0, the steady-state current was absent but not a transient flash-induced photoelectric signal (*13*). A transient charge displacement of comparable magnitude was observed in the early stages of the photocycle both at high and low pH, but integration of the transient photocurrent indicated net charge transfer at high (*15*) but not at low (*13*) pH. Control experiments had shown that the slight downward drift during illumination at pH 5.0 was from heating.

Measurement of Proton Transport in Proteorhodopsin-Liposomes. The simplest and most direct demonstration of light-driven transport, widely used for bacteriorhodopsin, is measurement of pH change with a glass electrode in a suspension of reconstituted liposomes. Figure 5 presents traces for light-induced pH changes from illuminated proteorhodopsin-containing liposomes. The illumination induced a net acidification, corresponding to protons being pumped from the inside to the outside of the liposomes, as described earlier (*1*, *4*). This is unlike in bacteriorhodopsin-containing liposomes, which cause net alkalinization because of a reverse orientation of the protein in liposomes as compared with the original cell membranes (*26*). As a control, we tested wild-type bacteriorhodopsin incorporated into liposomes also, and the well-known net alkalinization, with a similar time-course to the traces in Figure 5, was recorded (data not shown). To distinguish between two alternatives, (i) opposite orientation of proteorhodopsin in liposomes as compared with that of bacteriorhodopsin and (ii) opposite vectoriality of transport in proteorhodopsin and bacteriorhodopsin, the measurements were performed on suspensions of washed but intact *E. coli* cells in 100 mM NaCl (at pH 6.2, 7.4, and 8.5), with expressed proteorhodopsin after adding retinal. The cell suspensions produced net acidification under continuous illumination (data not shown), as membrane vesicles and reconstituted liposomes (*1*). This indicates that the direction of transport by the high pH photocycle is outward (i.e., the same as in bacteriorhodopsin); therefore, proteorhodopsin is incorporated into the liposomes with its cytoplasmic side facing the interior. As expected for a proton pump, addition of 50 μM of the uncoupler, CCCP (at pH 7.5), completely abolished the observed light-induced pH changes (data not shown).

Varying the pH produced pronounced effects on the magnitude and initial rate but not the sign of the light-induced pH changes. Figure 6 presents the steady-state number of protons pumped per illuminated proteorhodopsin molecule (Figure 6A) and the initial rate of pumping (Figure 6B). The data points are superimposed on the earlier reported titration curve of Asp-97 (*3*). The accuracy of the measurement is better for the steady-state amplitudes than for the initial rates (approximately ±10 vs ±19%, respectively), but the agreement with the titration curve is within error. It is clear that there is no residual transport, in either direction, as the pH is decreased to 4. The results indicate that proteorhodopsin pumps protons from the cytoplasmic to extracellular surface, and this transport, but not its vectoriality, depends on the protonation state of Asp-97. It reaches maximum rate at pH above 8 where Asp-97 is fully anionic and is able to act as the primary proton acceptor for the Schiff base proton, as we had proposed earlier (*3*). The transport observed in this

system correlates well with the pH-dependent amplitude of the M intermediate in the photocycle, as measured by time-resolved spectroscopy of the proteoliposomes (shown as open squares in Figure 6A).

Proteoliposomes with lipids extracted from purple membranes and with DOPC, the lipids used in the earlier study (*4*), gave similar results, and they are shown together in Figure 6. Lowering the pH to 4.1 did not result in irreversible changes, but raising the pH above 8.5 resulted in inactivation of the transport not reversed by lowering the pH. The high pH treatment did not affect the photocycle, however.

CONCLUSIONS

At pH low enough to keep the proton acceptor, Asp-97, protonated, photoexcitation of proteorhodopsin does not appear to result in proton transfers involving the retinal Schiff base and the two acidic residues, Asp-97 and Glu-108, that participate in transport at higher pH. Direct measurements with oriented membrane fragments and proteoliposomes revealed further that the light-driven proton transport is in the same direction at all pH, and its dependence on pH is consistent with the need to keep Asp-97 in the anionic state. The results contradict, therefore, the conclusions in ref *4* that transport occurs also at acid pH and in the opposite direction from the transport at higher pH.

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