Proton Uptake and Release Are Rate-Limiting Steps in the Photocycle of the Bacteriorhodopsin Mutant E204Q†

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ABSTRACT: In the absence of the putative proton release group, E204, the second half of the photocycle of the E204Q mutant of bacteriorhodopsin is slowed down more than 10-fold compared to the wild type. The effects of pH and D_2O on the M decay and O formation rates in E204Q suggest that proton uptake occurs concurrently with the N ↔ O transition, possibly coupled with the thermal reisomerization of the retinal. Hence, one of the rate-limiting steps in the slow E204Q photocycle is proton uptake from the outside medium, coincident with the decay of the slow component of M (the N ↔ O transition). The second rate-limiting step is the long lifetime of decay of the O state, due to a high activation barrier for the deprotonation of D85 in the O → bR step of the E204Q photocycle. Addition of the weakly acidic anions azide, cyanate, or formate accelerates the decay of the O intermediate, and restores the total photocycling time to that observed in the wild-type pigment, by accelerating the deprotonation of D85. We also find that azide similarly accelerates the decay of O in the wild type under conditions in which E204 does not deprotonate during the photocycle (pH < 6). It has previously been shown that azide and other weak acids can influence proton transfers in the cytoplasmic half of the protein [Tittor, J., Soell, C., Oesterhelt, D., Butt, H.-J., & Bamberg, E. (1989) *EMBO J. 8*, 3477−3482]; we suggest that these weak acids can affect proton transfers in the extracellular half of the protein as well.

Bacteriorhodopsin (bR)[1] is a light-driven proton pump found in the purple membrane of *Halobacterium salinarium*. Bacteriorhodopsin contains a retinal group, linked to the apoprotein at the ϵ-amino group of K216 *via* a protonated Schiff base. bR consists of seven transmembrane helices with the retinal roughly parallel to the plane of the membrane and located centrally in the pigment, thus dividing the protein into extracellular and cytoplasmic halves. Upon light absorption the chromophore isomerizes from the *all-trans* to the 13-*cis* configuration. After the initial photoisomerization, a series of thermal reactions follows during which the pigment passes through spectroscopically distinct intermediates termed K, L, M, N, O, and bR (the unphotolyzed state). The M intermediate is distinguished by an absorbance maximum (∼410 nm) that is substantially to the blue of the ground-state absorbance maximum (∼570 nm), while the absorbance of the O intermediate lies to the red (∼605 nm) of the ground-state pigment absorbance.

The photocycle of bR can be divided into two halves. The first half of the photocycle normally occurs in microseconds. It consists of photoisomerization and events leading up to and including the transfer of the Schiff base proton to the counterion, D85, and concomitant proton release into the extracellular medium from the proton release group, E204 (Brown et al., 1995a), during the L ↔ M transition. It is the deprotonated state of the Schiff base that causes the blue shift in the absorbance spectrum of M. The second half of the cycle is complete in tens of milliseconds and consists of reprotonation of the Schiff base by D96 in the M ↔ N transition, proton uptake from the cytoplasmic side and reisomerization of the chromophore in the N and O intermediates, and the recovery of the pigment in the O ↔ bR transition [for reviews of the photocycle, see Oesterhelt et al. (1992), Ebrey (1993), Khorana (1993), and Lanyi and Váró (1995)]. The final resetting of the system, i.e., the O ↔ bR transition, involves the deprotonation of D85 and the reprotonation of E204, which released its proton during the first half of the cycle. If, however, the proton release group is replaced with a neutral residue, or is unable to deprotonate in M because the bulk pH is lower than its pK_a, proton release occurs during the O ↔ bR step rather than during M (Zimányi et al., 1992; Govindjee et al., 1996). The proton appears in solution coincident with the decay of the O intermediate, suggesting that the deprotonation of D85 to the extracellular solution (rather than to E204) is associated with the O → bR transition in these situations (Gerwert et al., 1990; Govindjee et al., 1996).

We show here, using the E204Q mutant of bR, that the replacement of E204 with a neutral residue affects the events that occur in the second half of the photocycle. When E204 is replaced with glutamine, not only is proton release shifted from the M → N transition to the O → bR transition (Govindjee et al., 1996) but the time constants of the reactions following the reprotonation of the Schiff base (in

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[1] Abbreviations: bR, bacteriorhodopsin; WT, wild type. Some residues (e.g., D85) are designated using the one-letter code for amino acids. Mutant pigments are designated, e.g., E204Q, where the first letter and the number represent the wild-type residue and the second letter represents the substituted residue.

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the M → N transition) slow down by more than an order of magnitude. In E204Q, the N ↔ O transition, reflected as a slow decay component of M (Otto et al., 1989; Souvignier & Gerwert, 1992; Cao et al., 1993a), becomes slower with increasing pH or when D_2O instead of H_2O is used as the solvent. Formation of the O intermediate occurs simultaneously with this slow decay at all the pH values examined and in D_2O. These results suggest that the N ↔ O transition is rate-limited by the reprotonation of D96 and proton uptake from the outside medium. These results for E204Q contrast with a model proposed by Cao et al. (1993a) for the photocycle of the wild type at pH < 6, in which E204 remains protonated (uncharged) throughout the photocycle. Cao et al. propose that the N ↔ O transition should be identified with thermal reisomerization of the chromophore. They suggest that thermal reisomerization occurs much more rapidly at low pH (pH < 6) than at high pH, leading to proton uptake after formation of the O intermediate (after retinal reisomerization), rather than during the N ↔ O transition. In E204Q, however, proton uptake is coincident with the transition from N to O.

The completion of the entire E204Q cycle takes almost 1 s at neutral pH (Richter et al., 1996) compared to less than 50 ms in WT bR under similar conditions. The rate-limiting step is O decay (the deprotonation of D85 during the O → bR recovery). Millimolar concentrations of azide or other weak acids accelerate the O → R recovery, and thus the deprotonation of D85, in E204Q. Late proton release, which has been attributed to the millisecond release of the proton directly from D85 (rather than from E204) to the extracellular medium during the O ↔ bR transition (rather than the L ↔ M transition), is accelerated exactly to the same extent as the decay of the O intermediate. Azide also accelerates the decay of the O intermediate in the WT pigment at pH < 6, under conditions that are analogous to the E204Q mutation (E204 remains protonated throughout the photocycle). This shows that azide can affect the protonation/deprotonation reactions in the extracellular half of the proton channel as well as those in the cytoplasmic half (Tittor et al., 1989; LeCoutre et al., 1995; Brown & Lanyi, 1996).

MATERIALS AND METHODS

Site-directed mutagenesis of bR, and the transformation of the E204Q mutant into *H. salinarium* strain IV-8 were performed as described earlier (Balashov et al., 1993). The purple membrane was isolated using standard procedures (Oesterhelt & Stoeckenius, 1974). Flash-induced absorbance changes of the photocycle intermediates were measured according to previously described methods (Govindjee et al., 1990). Proton release and uptake was measured using pyranine as described earlier (Govindjee et al., 1996). Curve fitting was performed using our own programs and built-in features of the Kaleidagraph software package (Synergy Software, Reading, PA).

RESULTS

Correspondence between the Decay of M and the Formation and Decay of O in E204Q. The rate of formation of the blue-shifted M intermediate in the E204Q pigment is comparable to that in wild-type bR (data not shown). The decay of M in E204Q, however, is more than 10-fold slower than the wild type; we thus proceeded to characterize the E204Q M decay in further detail, focusing specifically on its pH dependence. As shown in Figure 1A, the decay of M is biphasic at neutral or lower pH but becomes triphasic at alkaline pH. The fastest decay component has a lifetime of 3−4 ms at all the pH values examined and represents the M ↔ N equilibration, as in the WT (Souvignier & Gerwert, 1992; Cao et al., 1993a). It comprises only ∼30% of the total M amplitude and is relatively independent of pH in the range (pH 3−9) we examined. As the M ↔ N transition involves the intramolecular transfer of a proton between D96 and the Schiff base (see reviews), the lack of a dependence of the fastest M decay component on pH suggests that neither D96 nor the Schiff base is being titrated in the pH range we examined. The fact that the fastest M decay component accounts for a relatively small fraction of the total M amplitude suggests that the pK_a difference between D96 and the Schiff base favors the former during the initial M ↔ N equilbrium. This is in contrast to the wild type, in which 70−80% of M decays as the fast component at pH < 9 (Cao et al., 1991; see Discussion).

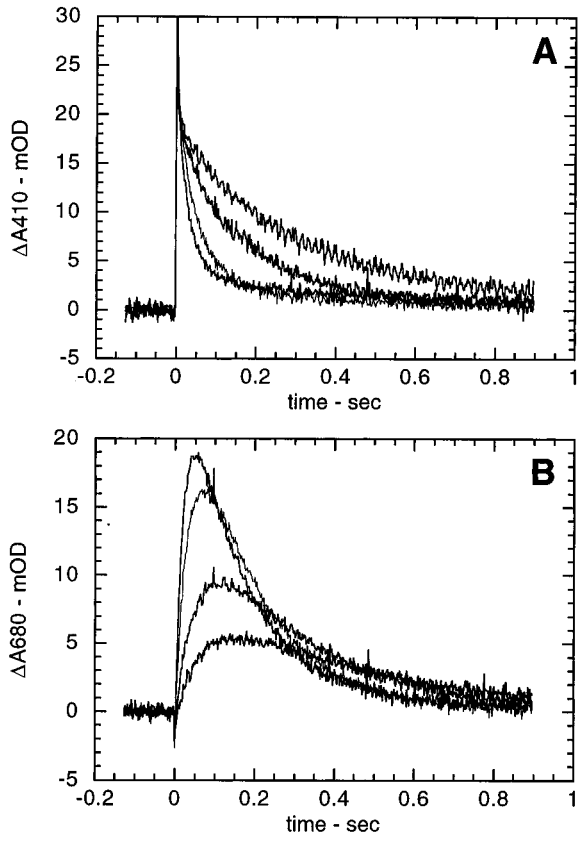

FIGURE 1: Flash-induced absorbance change at 410 nm due to the M photointermediate and at 680 nm due to the O photointermediate in the E204Q mutant. (A) M decay at pH 7.0, 7.5, 8.0, and 8.5 (fastest to slowest). (B) O formation and decay at pH 7.0, 7.5, 8.0, and 8.5 (highest to lowest peak amplitude). $\lambda_{actinic}$ = 532 nm; 140 mM KCl and 10 mM phosphate buffer, 20 °C; sample OD ∼0.5.

The lifetime of the second, slower M decay component coincides with the rise in absorbance at 680 nm, due to the formation of the O intermediate (Figure 1B). This correspondence holds between pH 3 and 9 (Figure 2). A third M decay component appears above pH 7 (Figure 2); its lifetime is equal to the lifetime of the O decay in this pH range (hundreds of milliseconds). The rate of the second M decay component, coinciding with O formation, exhibits a strong dependence on pH. As shown in Figure 2, the rate decreases with decreasing proton concentration in the bulk essentially linearly [slope ∼−0.4, rather than −1, probably

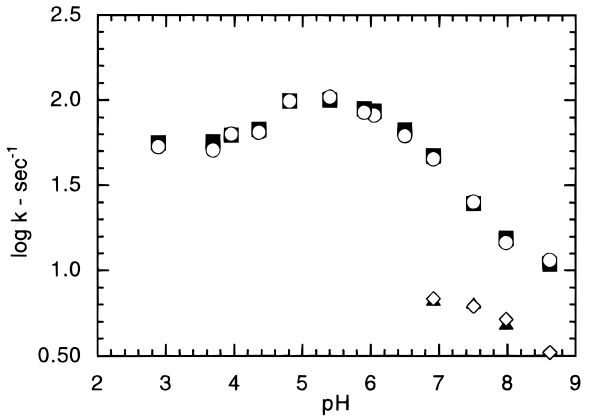

FIGURE 2: Rate constant of the second decay component of M (■) and formation of O (○) and the third decay component of M (▲) and decay of O (◇) at different pH values. The rate constants of the M decay were calculated by fitting traces such as those shown in Figure 1 to three decaying exponentials. The amplitude of the third (slowest) exponential is negligible below pH 7. Sample conditions were as in Figure 1.

due to an influence of the membrane surface charge; see Lanyi and Váró (1995)] between pH 6 and 8.5, deviating from linearity as a third M decay component becomes evident. As M partially decays before any O is formed, we infer that N (rather than O) accumulates initially, in analogy with the known intermediates of the WT photocycle. Since the second decay component of M coincides with the rise of O, we suggest that it reflects the decay of an M ↔ N equilibrium to O (i.e., the N ↔ O transition). The fact that both the second M decay component and the formation of O slow down equivalently with decreasing proton concentration in the bulk suggests that proton uptake from the bulk (and subsequent protonation of D96) is a rate-limiting step governing the N ↔ O transition. Further evidence for this suggestion is presented in Figure 5 (see below), which shows that proton uptake from the bulk, measured using a pH-sensitive dye, is coincident with the formation of O. To further examine the correspondence between proton uptake and the N ↔ O transition, we examined the decay of M (Figure 3A) and the formation of O (Figure 3B) in H_2O and D_2O under otherwise equivalent conditions (at pH 6.5). M decay in both situations was biexponential, with both components slowing down approximately 3-fold in D_2O compared to H_2O. O formation was also approximately 3 times slower in D_2O. Since the first component of M decay is representative of the equilibration of a proton between Asp96 and the Schiff base, we expected this component to be slower in D_2O. We interpret the observations that the second M decay component and O formation both slow down (to the same extent) in D_2O, while remaining coincident, as further suggesting that the N ↔ O transition in the E204Q mutant is rate-limited by proton uptake, at least above pH 6.

The rate of the second component of M decay saturates near pH 5 (Figure 2), suggesting that the bulk proton concentration is not rate-limiting for the N ↔ O step at this or lower pH values. Subramaniam and co-workers have used background light to accelerate reisomerization of the retinal from 13-*cis* to *all-trans* and speed up the decay of the (normally slowly decaying) O intermediate of the L93T mutant (Delaney et al., 1995; Delaney & Subramaniam, 1996). We wished to determine whether the rate-limiting factor in the N ↔ O transition in E204Q at low pH might

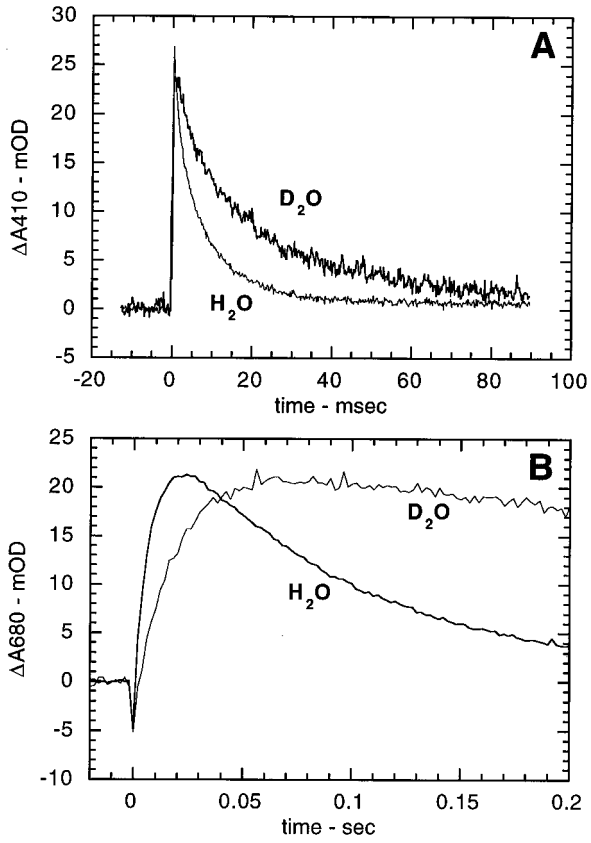

FIGURE 3: Effects of D_2O on the second half of the E204Q photocycle. (A) Biphasic decay of the M intermediate in H_2O and D_2O. (B) Formation and decay of the O intermediate in H_2O and D_2O. Both components of M decay and the formation of O slow down approximately 3-fold in D_2O. The second component of M decay and the formation of O remain coincident in D_2O. Traces obtained from samples in D_2O were normalized to the amplitude of the traces obtained from H_2O-soaked samples. Samples at pH/pD 6.5. $\lambda_{actinic}$ = 532 nm; 150 mM KCl, 20 °C; sample OD ~0.3.

similarly be the thermal reisomerization of the retinal in N. The presence of background green light (490−580 nm) coinciding with the absorbance of N did not, however, relieve the saturation or extend the linear region of the rate dependence of the N ↔ O transition to lower pH values (data not shown). As the background light was strong enough to change the fraction of photocycling pigment (see next section), we rule out the possibility that the lack of an effect was due to low background light intensity. This result suggests that the reisomerization of the retinal is not rate-limiting for the N ↔ O transition, even below pH 6. It has been proposed that D96 is not protonated directly from the bulk but rather that several other residues (possibly including R227, T46, and D38) may act as intermediaries, capturing a proton at the surface and donating it to D96 (Brown et al., 1994; Riesle et al., 1996). It is possible that the specific mechanism of D96 protonation, rather than the availibility of bulk protons, becomes rate-limiting at low pH. Interestingly, when the pH is decreased below 5, the second decay component of M slows down monotonically (from ~10 ms at pH 5 to ~18 ms at pH 2.9; see Figure 2), suggesting that the D96 protonation mechanism is being influenced by a pH-dependent process, possibly the titration of a participating residue.

It is thus likely that the second M decay component corresponds to the N ↔ O equilibration and reflects the uptake of a proton from the cytoplasm and the (eventual) reprotonation of D96. Since the third M decay component,

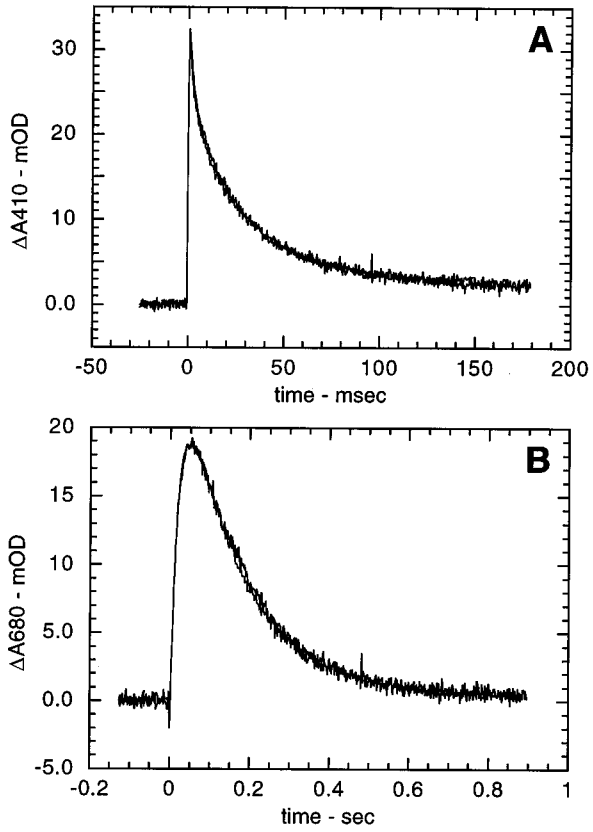

FIGURE 4: Flash-induced absorbance changes of the M intermediate (panel A) and of the O intermediate (panel B) in the absence and presence of background illumination of $\lambda \approx 410-570$ nm for the M intermediate and $\lambda > 620$ nm for the O intermediate. Traces were normalized to amplitudes measured in samples without background light. Samples were at pH 7.0. $\lambda_{actinic} = 532$ nm; 1 M NaCl / 25% glycerol, 20 °C, sample OD ~0.3.

which is present only at neutral or higher pH, coincides with the decay of O, it most probably reflects the existence of an O → N back-reaction at alkaline pH, resulting in an M ↔ N ↔ O equilibrium mixture which decays into bR. The evidence above suggests that the limiting event in the N → O step is proton uptake and the reprotonation of D96; the presence of an O → N back-reaction starting at pH 7 suggests that, as the pH is increased, it approaches the pK_a of a residue participating in one or both of these processes.

Effect of Background Illumination and Weak Acids on Decay of the O Intermediate in E204Q. The decay of the O intermediate is 1−2 orders of magnitude slower in E204Q than in the WT, with a lifetime of ~140 ms at neutral pH (Figures 1 and 2). As stated in the introduction, the O ↔ bR transition in E204Q involves the deprotonation of D85 into the bulk solution (Gerwert et al., 1990; Govindjee et al., 1996). We also considered the possibility that a second intramolecular event, thermal reisomerization of the retinal, might be taking place during the lifetime of the O intermediate, preceding proton release from D85. As stated in the previous section, red background light is able to accelerate the decay of the normally long-lived O intermediate in the L93T mutant, ostensibly by increasing the rate of retinal reisomerization above the thermal level (Delaney et al., 1995; Delaney & Subramaniam, 1996). We used the same method to determine whether thermal reisomerization is the rate-limiting factor in O decay in E204Q. As shown in Figure 4, providing background illumination ($\lambda \approx 410-570$ nm) during flash photolysis did not alter the kinetics of M formation or decay. The only observed effect was a small

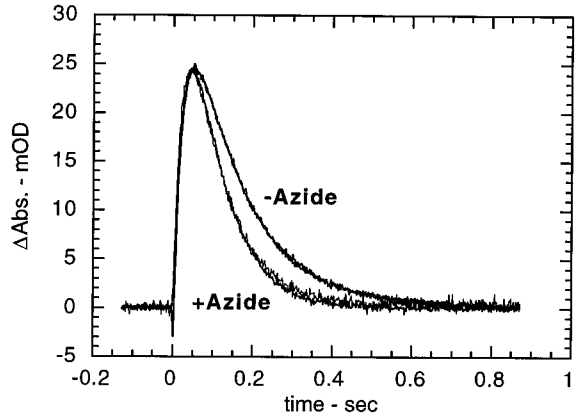

FIGURE 5: Flash-induced absorbance changes in E204Q mutant, showing a comparison of the kinetics of the O intermediate (at 680 nm) and proton uptake and release as measured with pyranine (at 460 nm), in the presence and absence of 4 mM azide. All the traces have been normalized at the maximum. Note that ΔA pyranine (proton uptake and release) coincides with ΔA 680 nm (formation and decay of O intermediate both in the absence and in the presence of azide). Samples were at pH 7.0. $\lambda_{actinic} = 532$ nm; 150 mM KCl, 20 °C; sample OD ~0.3.

(~3−5%) reduction in the M amplitude, probably due to photocycling of the pigment caused by the background light. In addition, background illumination in the red ($\lambda > 620$ nm) did not affect the kinetics of either the formation or the decay of O, only causing a small decrease in the amplitude. This suggests that the long decay lifetime of O is not due to a slow rate of thermal retinal reisomerization; since the background illumination was strong enough to cause some photocycling in the pigment, it was assumed that the absence of an effect on the photocycle kinetics was genuine and not due to low background light intensity.

Having eliminated thermal reisomerization of the retinal as a cause of the long decay time of O in E204Q, we investigated the remaining possibility that the slow rate of the O → bR transition in E204Q is caused by a slow rate of deprotonation of D85. Azide has been shown to accelerate both reprotonation and deprotonation kinetics of the Schiff base in the D96N mutant (Tittor et al., 1989, 1994). At neutral pH, addition of 4 mM azide has almost no effect on the kinetics of the M intermediate. Likewise, the formation of the O intermediate is unaffected, but its decay is accelerated almost 2-fold from ≈140 ms to ≈80 ms (Figure 5). In the presence of 4 mM azide the rate of proton uptake remains almost unchanged, whereas the lifetime of proton release also decreases from ≈140 ms to ≈80 ms, again coinciding with the rise and decay of the O intermediate (Figure 5). The acceleration of O decay (calculated as the difference in the decay constant with and without azide) varies linearly with the azide concentration between 0.1 and 20 mM (Figure 6). At higher concentrations, the effect of azide saturates; the O decay rate approaches the O formation rate asymptotically. This suggests that the effect of azide is dependent on the low pK_a of D85 in the O state, as the pK_a of D85 is too high in M and N to allow it to deprotonate. Somewhat surprisingly, the effect of azide (pK_a ~4.7) is essentially the same at pH 4.2, 5.2, and 7.0 (Figure 6B).

The two other weak acids that were tested, cyanate and formate, also accelerated the decay of O. Figure 7A,B shows that, at neutral pH, the rate of O decay increases with increasing concentration of cyanate and formate, while there is virtually no effect on the rate of O formation (data not shown). Surprisingly, at low pH (~3.9) there is a 3−4-fold

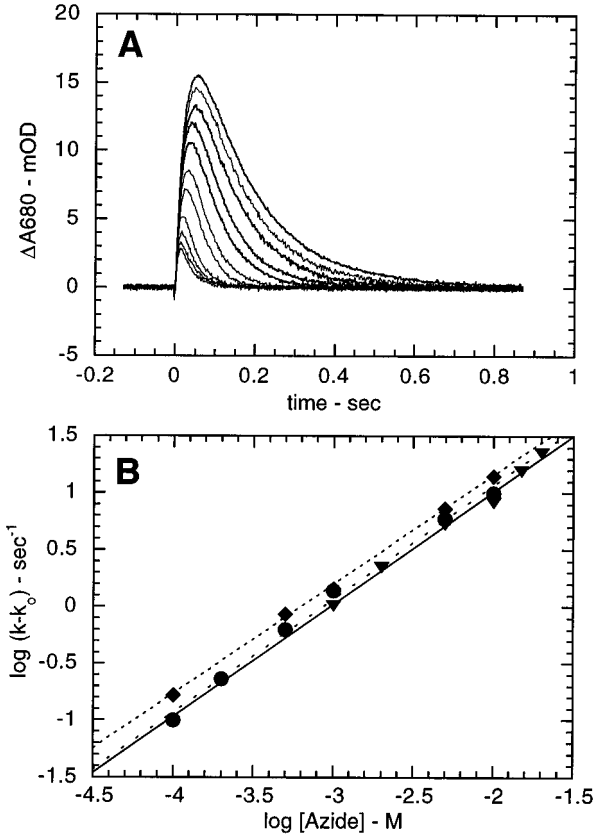

FIGURE 6: Addition of azide accelerates the decay of the O intermediate in E204Q. (A) Effect of increasing concentration of azide on the kinetics of the O intermediate in E204Q mutant. Traces shown are for 0, 1, 2, 5, 10, 15, 20, 50, 70, 90, and 100 mM added azide. (B) Log of the acceleration of O decay (rate in the presence of azide minus the rate in the absence of azide) plotted against the log of azide concentration at pH 4.2 (●), 5.2 (◆), and 7.0 (▼). Conditions were as in Figure 3 with the addition of 10 mM phosphate buffer at pH 4.2 and 5.2 and 10 mM Bis-tris propane buffer at pH 7.0.

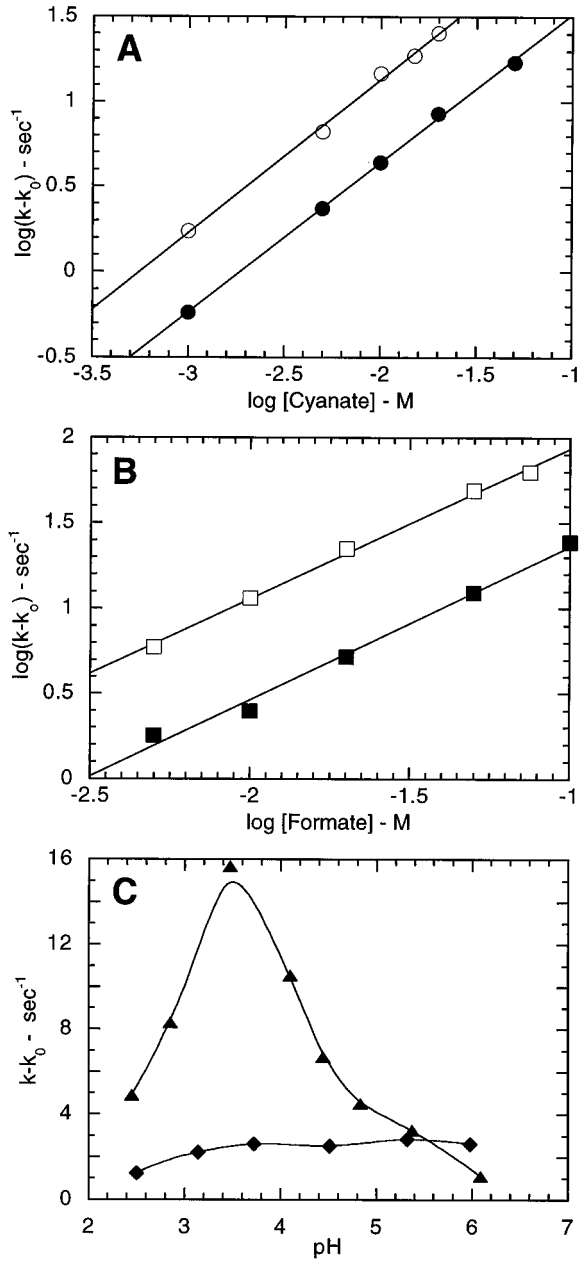

FIGURE 7: Effects of millimolar concentrations of cyanate and formate on the decay of the O intermediate. The log of the acceleration of O decay plotted against the log of cyanate (panel A) and formate (panel B) concentration at pH 7.0 (closed symbols) and 3.9 (open symbols). (Panel C) pH dependence of the acceleration of O decay by 10 mM formate at 150 mM (140 mM KCl and 10 mM phosphate; ▲) and 1.5 M (1.45 M KCl and 50 mM phosphate; ◆) salt concentration. The lines shown are merely to guide the eye and do not represent curve fits. Conditions: $\lambda_{actinic}$ = 532 nm; 20 °C; sample ODs ~0.3.

increase in the rate of O decay compared to that at pH 7.0. These results, as well as the relative lack of a pH dependence found for azide, appear to contradict the intuitive idea that the weak acids function as acceptors of the proton from Asp85, since the deprotonated forms of cyanate and formate (pK_as 3.6−3.8) are present at lower concentrations at pH 3.9 than at pH 7. We proceeded to examine the pH dependence of the acceleration of O decay caused by 10 mM formate at 150 mM and 1.5 M salt concentration (Figure 7C). For the data in 150 mM salt, the points above pH 4 can be fit with a shallow titration curve (n = 0.5−0.7; pK_a 3−4), superficially suggesting that it is the protonated form of the acids which catalyzes deprotonation of D85. However, the formate effect decreases below pH 4, at which the protonated form dominates. This points to an influence of the membrane surface charge (possibly on the accessibility of the weak acids) as the cause of the pH dependence above pH 4, and the actual titration of the acid molecules as the cause of the pH dependence below pH 4. The former effect should not be evident at high salt concentrations, due to shielding of the membrane surface charges by ions. Indeed, at 1.5 M KCl, the acceleration of O decay caused by 10 mM formate is pH-independent up to pH 3 (Figure 7C), below which the formate probably becomes protonated. The data thus suggest that it is the deprotonated form of formate which is active, possibly acting as a proton acceptor from D85 (see Discussion). The anomalous pH dependence of the formate effect (at least at low salt concentration) appears to arise from the changing accessibility of the (negatively charged) acid species to the protein, due to the titration of charges on the purple membrane surface.

The temperature dependence of O decay in the absence and presence of 10 mM azide, as well as their difference (the azide effect itself), is shown in Figure 8. It is apparent that azide accelerates O decay by making the activation entropy for D85 deprotonation less negative [−45 J/(mol K) rather than −140 J/(mol K)]. The activation enthalpy is actually larger for the azide-catalyzed deprotonation than the normal deprotonation of D85 (51 kJ/mol compared to 25 kJ/mol).

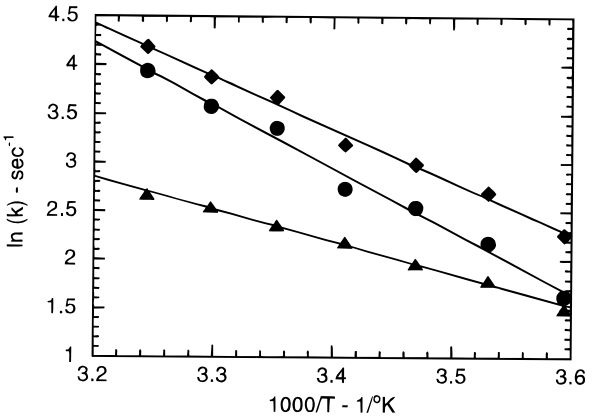

FIGURE 8: Temperature dependence of the rate of O decay in the absence and presence of 10 mM azide. Rates shown are for O decay without azide (▲), O decay with azide (◆), and the difference between the two (rate with azide minus rate without azide, ●). The data were fit with the Eyring equation, $K = kT/h \exp(\Delta S^{\ddagger}/R) \exp(-\Delta H^{\ddagger}/RT)$, where K = decay rate constant, $\Delta S^{\ddagger}$ = activation entropy, $\Delta H^{\ddagger}$ = activation enthalpy, T = absolute temperature, k = Boltzmann constant, h = Planck's constant, and R = ideal gas constant. The fits yield the following values: O decay without azide, $\Delta H^{\ddagger} = 25$ kJ/mol and $\Delta S^{\ddagger} = -140$ J/(mol K); O decay with 10 mM azide, $\Delta H^{\ddagger} = 43$ kJ/mol and $\Delta S^{\ddagger} = -72$ J/(mol K); azide effect, $\Delta H^{\ddagger} = 51$ kJ/mol and $\Delta S^{\ddagger} = -45$ J/(mol K). Conditions: pH = 6.8; $\lambda_{actinic} = 532$ nm; sample ODs ~0.3.

Effect of Azide on the Decay of the O Intermediate in WT bR. To investigate whether the effect of azide on the decay of O is exclusive to E204Q, we performed a similar experiment on samples of WT purple membrane. In the WT photocycle, O accumulates maximally at pH < 6, under conditions in which E204 remains protonated throughout the photocycle. As stated previously, the WT photocycle under these conditions is similar to that of E204Q in that proton release occurs after proton uptake, during the O → bR transition. As shown in Figure 9A, azide accelerates the decay of O in the WT (at pH 4.7) as well as in E204Q. The dependence between the azide concentration and the acceleration of O decay remains linear in the WT but has a slope of ~0.55 (Figure 9B) rather than ~1, as found in E204Q. The reason for this discrepancy is not obvious, as there should be no difference in surface charge between the WT and E204Q under the experimental conditions used.

The temperature dependence of the rate of O decay in the WT (Figure 9C) shows that it occurs more rapidly than in E204Q primarily because of a less negative activation entropy [−98 J/(mol K) rather than −140 J/(mol K)]; the activation enthalpy is higher in the WT (34 rather than 25 kJ/mol). As in E204Q, azide accelerates O decay by increasing the activation entropy. The activation energetics of the azide effect were found to be essentially the same in the WT as in E204Q [$\Delta H^{\ddagger}$= 52 kJ/mol vs 51 kJ/mol in E204Q, $\Delta S^{\ddagger}$= −47 J/(mol K) vs −45 J/(mol K) in E204Q], suggesting that azide catalysis of D85 deprotonation occurs identically in both proteins. The equilibration of azide into the extracellular half of the protein can apparently occur generally and is not merely allowed or strongly affected by the E204Q mutation; the effect of the mutation appears primarily to influence the base rate of D85 deprotonation.

We also examined the effect of azide on the decay of O in the WT at pH 7, at which E204 deprotonates in M and is reprotonated (probably directly from D85) during the O ↔ bR transition. Addition of up to 100 mM azide had no observable effect on O decay, suggesting that azide does not effectively influence the proton transfer between D85 and E204 (data not shown).

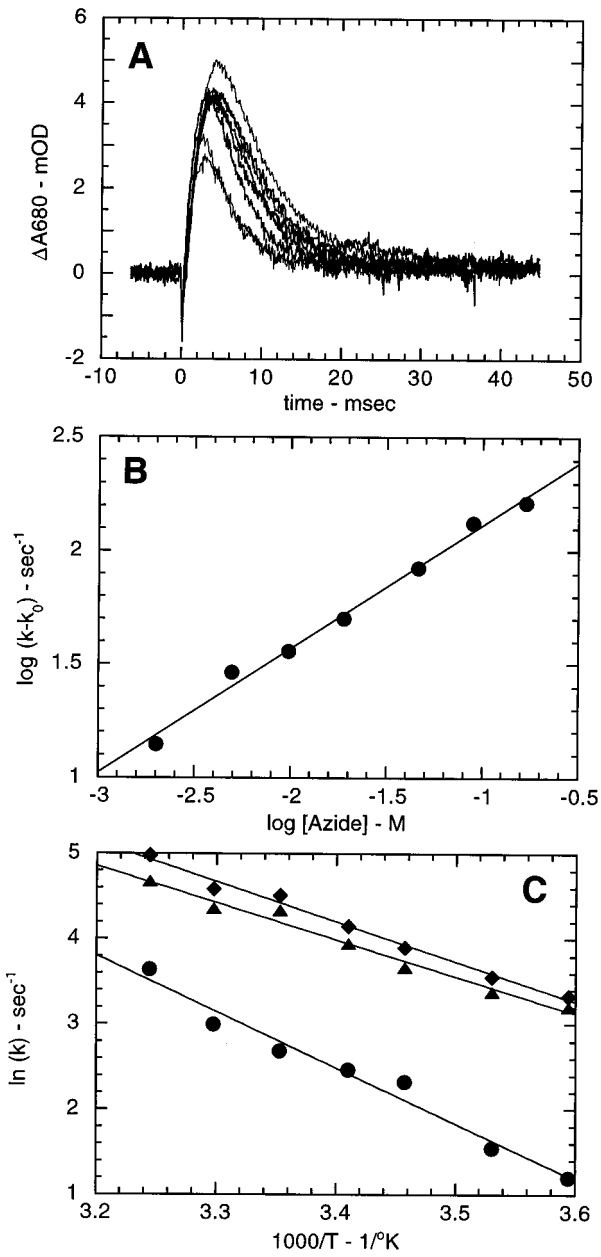

FIGURE 9: Addition of azide accelerates the decay of the O intermediate in the WT. (A) Effect of increasing concentration of azide on the kinetics of the O intermediate in WT bR. Traces shown are for 0, 2, 4.9, 9.8, 19, 46, 89, and 169 mM added azide. (B) Log of the acceleration of O decay (rate in the presence of azide minus the rate in the absence of azide) plotted against the log of azide concentration. (C) Temperature dependence of the rate of O decay in the absence and presence of 20 mM azide. Rates shown are for O decay without azide (▲), O decay with azide (◆), and the difference between the two (rate with azide minus rate without azide, ●). The data were fit as described for Figure 8, yielding the following values: O decay without azide, $\Delta H^{\ddagger} = 34$ kJ/mol and $\Delta S^{\ddagger} = -98$ J/(mol K); O decay with 20 mM azide, $\Delta H^{\ddagger} = 37$ kJ/mol and $\Delta S^{\ddagger} = -86$ J/(mol K); azide effect, $\Delta H^{\ddagger} = 52$ kJ/mol and $\Delta S^{\ddagger} = -47$ J/(mol K). Samples were pH 4.7. $\lambda_{actinic} = 532$ nm; 100 mM KCl and 50 mM phosphate buffer; sample ODs ~0.3.

DISCUSSION

Substitution of E204 with a neutral residue, glutamine, increases the photocycling time over 10-fold (Brown et al., 1995a; Richter et al., 1996). The time constants in the so-called first half of the photocycle, including the intermediates

K, L, and M, are relatively unaffected by the mutation. The time constants of the reactions in the second half, however, are considerably slowed down; thus, we have focused our investigation on these processes. There are four main events that occur during the second half of the photocycle: (1) reprotonation of the Schiff base by D96 in the M ↔ N transition, (2) proton uptake from the outside medium and the reprotonation of D96, (3) thermal reisomerization of the retinal chromophore from 13-*cis* to *all-trans*, and (4) deprotonation of D85 and the final recovery of the pigment in the O → bR transition (see reviews referred to in introduction). Any one or a combination of steps could be the rate-limiting step(s) in the photocycle of the E204Q pigment. Our results suggest that, in E204Q, proton uptake from the cytoplasmic solution limits the transition of N to O, while deprotonation of D85 to the extracellular solution limits the decay of O into bR.

Relationship between M and O Intermediates and Nature of the N ↔ O Transition in E204Q. The decay of the M intermediate in the WT is biphasic below pH 10; the two decay components have been suggested to reflect the M ↔ N and N ↔ O equilibria, respectively (Otto et al., 1989; Gerwert et al., 1990; Cao et al., 1991). In E204Q, M decay is similarly biphasic up to pH 7. The rates of the second M decay component and the rise of the O intermediate are identical (Figure 2); in addition, these rates vary linearly with the bulk proton concentration between pH 6 and 8, suggesting that the rates are limited by the external proton concentration in this range. Measurements with pH-sensitive dyes also show that O formation and proton uptake coincide (Figure 5). We thus assign the fastest component of the M decay ($\tau \approx 3-4$ ms) to the M ↔ N equilibrium and the second component to the N ↔ O transition, in correspondence with the WT photocycle. However, both of these M decay components exhibit features in E204Q that differ from the corresponding components in the wild type.

The relatively small amplitude (~30% of the total signal) of the fastest M decay component in E204Q suggests that the protonation equilibrium between D96 and the Schiff base is biased toward D96 by the equivalent of 0.3−0.4 pK_a unit. This is contrary to the situation in the WT, in which a greater portion of M decays as the fast component (Cao et al., 1991). The pK_a of D96 has been proposed to drop to 7 or below at this point in the wild-type photocycle (Cao et al., 1993b), while the pK_a of the Schiff base in N (in the D96N mutant) was recently measured to be ~8.2−8.3 (Brown & Lanyi, 1996). The difference between these pK_a values is consistent with the experimental evidence that the M ↔ N equilibrium favors N (the protonation equilbrium favors the Schiff base over D96) by 4−5-fold in the WT. In E204Q, the equilibrium favors M (protonation equilibrium favors D96) approximately 2-fold, suggesting that the mutation either prevents the reduction of the D96 pK_a or the increase of the Schiff base pK_a after the M intermediate has formed.

Lanyi and co-workers have found that in wild-type bR, proton uptake occurs after formation of the O intermediate (as a transition between substates of O) at pH < 6 and during the N intermediate (as a transition between substates of N) at pH > 6 (Cao et al., 1993a; Zimányi et al., 1993). The dividing line between the two pH regions is the pK_a of the proton release group, E204, while the pigment is in the M intermediate. To account for the differing sequence between the (spectroscopically observed) N ↔ O transition and the proton uptake (measured using pH-sensitive dyes), these authors have suggested that different electrostatic environments of the Schiff base and retinal in the two pH ranges, linked to two different charge states of E204 in the second half of the photocycle, lead to different rates of thermal reisomerization (with the reisomerization rate being faster at low pH) (Lanyi & Váró, 1995). The spectroscopic N ↔ O transition is thus identified with the thermal reisomerization of the retinal. However, recent experiments of Subramaniam and co-workers (Delaney et al., 1995; Delaney & Subramaniam, 1996) suggest that the N ↔ O transition can occur without retinal reisomerization. They found evidence for the presence of an O substate with a 13−*cis* chromophore in the L93T and D96N/L93T mutants, suggesting that retinal reisomerization can take place after formation of O, as had been suggested by Ebrey (1993).

We expected that the behavior of E204Q would be similar to that of the WT at low pH (pH ≈ 5), in which the E204 residue remains protonated throughout the photocycle and proton release is directly from D85 during the O → bR step. However, in E204Q, we have found a third case distinct from the two mentioned above. In this mutant, the N ↔ O transition is rate-limited under each condition we examined by proton uptake and reprotonation of D96. Retinal reisomerization is not a rate-limiting step under the conditions we examined. It is not possible to determine, however, whether the spectroscopically observed N ↔ O transition should be identified with the proton uptake and associated reprotonation of D96. It is equally likely that reprotonation of D96 is closely coupled with retinal reisomerization in this mutant, so that the two events are kinetically inseparable. Hence, it is possible that the spectroscopic N ↔ O transition should still be identified with retinal reisomerization, and that the apparent dependence of the N ↔ O transition rate on proton concentration and the effect of D_2O actually reflects a limitation of the retinal reisomerization rate by these factors in the E204Q mutant.

It has been suggested that the barrier for thermal isomerization is greatly lowered when D96, D85, and the Schiff base are protonated, as is the case in O or, by definition, in late substates of N after proton uptake from the cytoplasm has occured (Balashov et al., 1993, 1995; Delaney & Subramaniam, 1996). This provides a basis for the suggestion (for both the WT and E204Q) that reprotonation of D96 enhances the retinal reisomerization rate. It is possible, however, that the reisomerization rate in the WT is already relatively high and is enhanced only marginally by reprotonation of D96. Hence, it would be possible to observe retinal reisomerization both before proton uptake (at pH < 6) and after proton uptake (at pH > 6) in the WT, as reprotonation of D96 would not play a large part in determining the observed reisomerization rate; perhaps, the influence of E204 would be more significant (Lanyi & Váró, 1995). In contrast, it is possible that the E204Q mutation alters the electrostatic environment of the Schiff base and the retinal or affects the dynamics of the protein in such a manner as to cause a lower base rate of thermal reisomerization in N. The enhancement of the reisomerization rate contributed by reprotonation of D96 would be more significant in E204Q than in the WT, and retinal reisomerization would be more strongly coupled to proton uptake. Interestingly, the rate of thermal isomerization of the chromophore in the unphotolyzed state (rate of dark adaptation) is slower in E204Q than in the WT at neutral pH but not at acid pH;

however, the state of the protein under these conditions may not be analogous to that in the N and O intermediates.

A physical mechanism by which the effects of E204 could be transduced to the rest of the protein, including the cytoplasmic side, is the conformational change that has been suggested to take place during the M ↔ N transition (Subramaniam et al., 1993; Brown et al., 1995b; Váró & Lanyi, 1995). The unusual equilibrium between M and N (protonation equilibrium between D96 and the Schiff base) shifted toward the former in E204Q, mentioned at the beginning of this section, could be the byproduct of a defect in this conformational change caused by the E204Q substitution. Another piece of evidence which suggests that this conformational change is defective in E204Q comes from the slow rate of proton uptake on the cytoplasmic side in the mutant. It has been suggested that the rates of protonation, from the solvent, of the exposed cytoplasmic proton uptake groups in bR is very fast, on the order of 10^{10} M^{-1} s^{-1} (Nachliel et al., 1996). This corresponds to rate constants of 10^3-10^4 s^{-1} at pH 6−7, agreeing with the evidence that proton uptake is not a rate-limiting step in the photocycle of wild-type bR. In E204Q, however, the observed protonation rates are only about 10^2 s^{-1} or less. This suggests that the usual proton uptake groups are not as exposed to the cytoplasmic solvent in E204Q, which could result from a defect in a conformational change which normally causes these residues to be exposed. Similarly, such a defect could be transduced to the Schiff base and retinal binding pocket, leading to thermal reisomerization that is dependent on other factors in E204Q than in the WT.

An interesting phenomenon in E204Q is that the rate of the second decay component of M (i.e., the N ↔ O transition) saturates with increasing bulk proton concentration below pH 6. This suggests that the proton capture mechanism, rather than the availibility of protons at the surface, becomes limiting for the protonation of D96. It has been suggested (Brown et al., 1994; Riesle et al., 1996) that several residues, including T46, R227, and/or D38, form a complex of interacting residues that can capture a proton from the surface and direct it toward D96. The limiting rate of the N ↔ O transition changes slightly but monotonically between pH 5 and 3, possibly due to a change in the protonation state of one of these groups.

Acceleration of O Decay by Weak Acids. In E204Q, O accumulates even at high pH when the decay of N is slowed down due to the low bulk proton concentration. This is due to the slow decay rate of O itself. As the presence of red background light does not accelerate the decay of O, the slow decay rate is not due to accumulation of a 13-*cis* form of O, such as was observed in the L93T mutant (Delaney et al., 1995; Delaney & Subramaniam, 1996).

Azide has been proposed to directly aid the reprotonation of the Schiff base in the D96N mutant, where the photocycle is arrested in the M state due to the absence of D96 (Tittor et al., 1989, 1994; Cao et al., 1993a). It was suggested that azide acts by shuttling protons from the outside medium directly to the Schiff base. However, on the basis of evidence from single and double mutants of D85 and D96, Le Coutre et al. (1995) suggested that azide binds in the extracellular channel near D85 but accelerates the reprotonation of the Schiff base in D96N by restoring the hydrogen-bonded water structure between the Schiff base and D96.

In E204Q as well as the WT at pH < 6, we show that azide and other weak acids can act in the extracellular channel to facilitate the deprotonation of D85 in the final step of O decay and pigment recovery. Since the pK_a of azide is ∼4.7, at neutral pH the azide anion is the predominant species; thus, it is tempting to suggest that N_3^- acts as a proton acceptor shuttling protons from D85 to the outside medium. However, lowering the pH to a value below the pK_a of azide results in a similar concentration dependence of the acceleration of O decay; there is virtually no difference between pH 4.2, 5.2, and 7.0 (Figure 6B).

Other weak acids such as cyanate and formate also accelerate the decay of O with a linear concentration dependence. In these cases, however, the acceleration of O decay exhibits a pH dependence in 150 mM KCl. The acceleration of O decay is approximately 3-fold greater at pH 3.9 compared to pH 7.0. As shown in Figure 7C, the pH dependence of O decay at a fixed concentration of formate (10 mM) shows a shallow ($n = 0.5-0.7$) titration curve with a pK_a of 3.5 ± 0.7, a value close to the pK_a of formate. This superficially implies that the active species is the protonated form and that the weak acids catalyze deprotonation of D85 through some other mechanism than merely by acting as recipients of the proton. However, with decreasing pH below 3.5, the effect of formate decreases as the fraction of the protonated species increases. This suggests that the pH dependence of the formate effect is actually due to a combination of two factors. Assuming that the active species is the deprotonated, negatively charged form of formate, it is possible that the decrease in (negative) surface charge as the pH is lowered leads to increasing accessibility of the deprotonated formate to the protein interior and to D85. However, as the pH is lowered further, this will be counteracted by the depletion in the concentration of the deprotonated (active) species and rise in the concentration of protonated formate. Data obtained from samples in 1.5 M KCl (Figure 7C), at which the influence of the surface charge on accessibility of formate to the membrane surface (and to the protein) should be much less significant, shows essentially no pH dependence above pH 3, supporting this model. In light of this result, the apparent lack of a pH dependence for the acceleration of O decay by azide may be ascribed to the fact that the negative charge on (deprotonated) azide may be delocalized over the entire molecule; thus, the accessibility of azide may not be as influenced by the membrane surface charge as formate or cyanate, and the effect of azide would not be pH-dependent above the pK_a of azide. This would agree with the known ability of azide to act as a protonophore. We thus suggest that the deprotonated, rather than protonated, forms of the weak acids we examined are active in catalyzing the deprotonation of D85, possibly by acting as proton acceptors themselves.

The temperature dependence of the azide effect (Figures 8 and 9C) suggests that azide increases the rate of deprotonation of D85 by increasing (making less negative) the activation entropy rather than by lowering the activation enthalpy. In wild type at low pH (in which E204 remains protonated throughout the photocycle), the deprotonation of D85 during the O → bR transition is much more rapid than in E204Q, suggesting that the absence of a negative charge on E204 is not the reason for the higher activation barrier for D85 deprotonation in the mutant. Comparison of the temperature dependence of O decay between the WT and E204Q shows that E204 itself influences the activation entropy rather than enthalpy of the deprotonation of D85, as do the weak acids in our experiments, perhaps through

its influence on the water molecules which have been suggested to mediate interactions between residues in the extracellular half of the proton channel (Humphrey et al., 1994).

The mutation of E204 to glutamine is not necessary to confer accessibility of azide to D85. We found that azide accelerates the decay of the O intermediate in WT purple membrane at pH < 6, in a situation analogous to the E204Q photocycle (Figure 9). The equilibration of weak acids between the solution and the extracellular half of the protein thus appears to be a general phenomenon and must occur on at least a millisecond time scale, since the O decay lifetime is approximately 10 ms even without azide present. Azide had no effect, however, on the decay of O in the WT at neutral pH; under these conditions, E204 deprotonates in M and proton transfer (probably) occurs at the end of the photocycle from D85 to E204 rather than from D85 into solution. The lack of an azide effect could be due to one of the following reasons: (1) the negative charge on E204 is sufficient to prevent access of azide to the protein environment near D85; (2) the proton transfer between D85 and E204 simply occurs in a manner that is not influenced by azide. Azide may catalyze proton transfer between D85 and the bulk through a (rapid) diffusion and encounter process but may be ineffective in affecting a directed transfer between the two fixed residues within the protein.

Conclusions. As would be expected, the substitution of the putative proton release group, E204, with a neutral glutamine residue alters the proton release behavior of bR, moving the important proton release event from the M intermediate to the O intermediate. The E204Q substitution, however, also exhibits surprising effects on the second half of the photocycle, influencing events that occur at sites located relatively far away from as well as near the 204 position. These combine to increase the total photocycling time of the mutant some 10-fold over the wild-type photocycling time. The E204Q substitution results, first, in a correlation between the spectroscopically observed N ↔ O transition and proton uptake and reprotonation of D96. Second, the substitution results in an increased activation barrier for the deprotonation of D85 during the O ↔ bR step, which can be relieved by azide and other weak acids. Azide is similarly effective in increasing the rate of the O ↔ bR transition in the WT at pH < 6, under conditions analogous to those in E204Q.

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