Relationship of Retinal Configuration and Internal Proton Transfer at the End of the Bacteriorhodopsin Photocycle†

Hans-Thomas Richter,‡ Richard Needleman,§ Hideki Kandori,|| Akio Maeda,|| and Janos K. Lanyi*,‡

Department of Physiology & Biophysics, University of California, Irvine, California 92697, Department of Biochemistry, Wayne State University, Detroit, Michigan 48201, and Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606-01, Japan

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ABSTRACT: In the last step of the bacteriorhodopsin photocycle the initial state is regenerated from the O intermediate in an essentially unidirectional reaction. Comparison of the rate of this photocycle step and the rate of deprotonation of Asp-85 in pH jump experiments with various site-specific mutants indicates that recovery of the initial state is influenced by (1) residues such as Glu-204 that affect deprotonation of Asp-85 and (2) residues such as Leu-93 that contact the retinal and therefore must affect its thermal reisomerization from 13-*cis* to *all-trans* as suggested by Delaney, Schweiger, and Subramaniam (*Proc. Natl. Acad. Sci. U.S.A. 92*, 11120−11124, 1995). These results, together with FTIR spectra (Kandori, Hatanaka, Yamazaki, Needleman, Brown, Richter, Lanyi, & Maeda, manuscript in preparation) of the last intermediate in the photocycles of representatives of the two kinds of mutants, E204Q and L93M, suggest the following sequence of events: reisomerization of the retinal from 13-*cis* to an *all-trans* configuration that contains a twisted chain (with high amplitude hydrogen out-of-plane vibrational bands) triggers proton transfer from Asp-85 to Glu-204 or directly to the extracellular surface, and the proton transfer in turn triggers relaxation of the twist in the retinal. The involvement of the proton transfer in the kinetics of this sequence suggests the reason for the unidirectionality of the overall reaction: upon reisomerization of the retinal the very low pK_a of Asp-85 in the unphotolyzed protein is reestablished and this residue thereby becomes a good proton donor.

Photoisomerization of the retinal of bacteriorhodopsin (BR[1]) from *all-trans* to 13-*cis* initiates a reaction cycle ("photocycle") that translocates a proton across the membrane of halobacteria [for reviews see Oesterhelt et al. (1992); Rothschild (1992); Lanyi (1993); Krebs and Khorana (1993); Ebrey (1993)]. The intermediate states that arise and decay have been termed J, K, L, M, N, and O. The first proton transfer in the transport is the protonation of the anionic Asp-85 by the retinal Schiff base (the L → M_1 reaction). At neutral pH protonation of Asp-85 triggers the release of a proton from Glu-204 to the extracellular surface (Brown et al., 1995; Richter et al., 1996). This step and the subsequent protein conformation changes are associated with a change of the connectivity of the Schiff base from Asp-85 to Asp-96 (the M_1 → M_2 reaction). Reprotonation of the Schiff base from Asp-96 near the cytoplasmic side (the M_2 → N reaction) is followed by protonation of Asp-96 from the cytoplasmic surface and reisomerization of the retinal to *all-trans* (Ames & Mathies, 1990; Smith et al., 1983), the N → O reaction. Asp-85 remains protonated until the recovery of the initial state in the last photocycle step (Bousché et al., 1992; Hessling et al., 1993; footnote 2), the O → BR reaction. As in other ionic pumps, the excess free energy gained upon energizing the system (in this case photoisomerization of the retinal) is thereby utilized to move a proton across the membrane and generate a transmembrane electrochemical proton potential. Clues to the flow of free energy as the proton is translocated in the reaction cycle have been provided by the temperature and pressure dependencies of the rate constants of the above described transformations (Váró & Lanyi, 1991b, 1995).

Many of the photocycle steps after formation of the K intermediate appear to be, or can be under some conditions, not far from equilibrium and proceed therefore without much change in free energy (Váró & Lanyi, 1991b). Under physiological conditions the excess free energy acquired in the photoreaction is dissipated mainly in three photocycle steps: (a) the release of a proton to the extracellular side with a pK_a < pH, during what is kinetically the M_1 → M_2 reaction (Zimányi et al., 1992); (b) the uptake of a proton at the cytoplasmic side with a pK_a > pH, during the lifetime of the N state (Zimányi et al., 1993); and (c) the final O → BR reaction that leads to the recovery of the initial state. The first two of these confer pH dependencies on the respective photocycle steps, and through these pH depend-

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* Author to whom correspondence should be addressed.

‡ University of California.

§ Wayne State University.

|| Kyoto University.

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[1] Abbreviations: BR, bacteriorhodopsin with *all-trans* chromophore; K, L, M, N, and O, photointermediates of the BR photocycle; site-directed mutants are described with the wild type and mutated residues separated by the residue number, e.g., E204Q or Y185F/E204Q; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

[2] Kandori, Hatanaka, Yamazaki, Needleman, Brown, Richter, Lanyi, & Maeda, manuscript in preparation.

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encies the pH on either side of the membrane will determine whether free energy is utilized more for proton release or proton uptake. The third of these steps has proved to be virtually irreversible under all conditions investigated and seems to dissipate the largest part of the excess free energy. In this sense, therefore, the O → BR reaction is the step that ensures the unidirectionality of the proton translocation and will overcome any back-pressure on the transport cycle from the electrochemical potential created for protons across the membrane. Little information has been available so far on what determines the rate of this reaction and what makes it irreversible.

When the rate of the reisomerization of retinal to *all-trans* is slow, as at high pH, the last intermediate that accumulates is the N state, in which the retinal is still 13-*cis* (Fodor et al., 1988) and little or none of the O state is observed. Under more usual conditions where the reisomerization is rapid, the O intermediate accumulates in measurable amounts before repopulation of the initial state. Asp-85 is still protonated in the O state, and O contains *all-trans*-retinal like the unphotolyzed protein but with large-amplitude HOOP bands that indicate a twisted configuration along the polyene chain. The red-shift of the maximum of O originates partly from the less complete compensation of the positive charge of the Schiff base when Asp-85 is protonated (Nakanishi et al., 1980) and probably partly also from the twist of the retinal chain like in the K intermediate (Braiman & Mathies, 1982; Rothschild et al., 1984). The O state is similar to acid blue bacteriorhodopsin in which Asp-85 is protonated because the pH is lower than its pK_a, although the retinal in this species is not distorted (Pande et al., 1985; Smith & Mathies, 1985). It resembles also a quasi-stable blue form that accumulates during illumination of the Y185F mutant (He et al., 1993; Rath et al., 1993). What determines the decay of the O intermediate to BR? In the L93A and L93T mutants, which have O states with particularly long lifetimes, chemical analysis of extracted retinal suggested recently that these O states contain 13-*cis*-retinal (Delaney et al., 1995; Delaney & Subramaniam, 1996). It was argued that its decay is slowed because the reisomerization to *all-trans* is perturbed by the residue replacement near the 13-methyl retinal group. This would occur in the Leu-93 mutants because protein conformational changes are coupled to retinal bond rotations through steric interaction of residue 93 and the 13-methyl group. Recent results indicate that the protein is coupled to the retinal also through interaction of Trp-182 and the 9-methyl group (Yamazaki et al., 1995; Weidlich et al., 1996).

We report here studies of the slowed decay of O in two kinds of mutants. In the first class, where the deprotonation of Asp-85 is retarded, the decay of the O state correlates well with the rate of the deprotonation of Asp-85 as measured by pH jump in the unphotolyzed proteins. In these cases the decay of O is limited by the deprotonation reaction. In the second class of mutants this correlation is absent, and FTIR spectra in studies to be reported elsewhere[2] indicate that decay of what is identified as the O state (but depending on its definition may be also called the N state) must be limited by reisomerization of the 13-*cis*-retinal. From these results we suggest a causal sequence for the events that lead to recovery of the initial state of the protein.

MATERIALS AND METHODS

The method of constructing the mutants E204Q, E204D, Y185F/E204Q, V49A, A53V, L93T, L93M, and L93S has been described before (Needleman et al., 1991). These proteins, and the wild type, were isolated as purple membranes by a standard method (Oesterhelt & Stoeckenius, 1974). Spectroscopic changes were measured either without photoexcitation after a pH jump in a stopped-flow spectrophotometer (Model SF-51, Hi-Tech Scientific Ltd, Salisbury, U.K., Kataoka et al., 1994) or after photoexcitation with a 7 ns laser flash at 532 nm as before (Brown et al., 1994b).

For the pH jump experiments the BR was washed several times in distilled H_2O, its concentration adjusted to ~1.5 OD, and light adapted. The contents of syringe "A", a purple membrane suspension with a low buffer concentration at the starting pH, and syringe "B", 100 mM Na_2HPO_4 at the desired final pH, were mixed, and absorption was followed at the indicated wavelengths.

RESULTS

Time-Resolved Titration of Asp-85. Proton exchange between Asp-85 and the aqueous phase occurs on a millisecond time scale (Druckmann et al., 1979, 1985, 1995). This reaction can be measured spectroscopically, because when Asp-85 is unprotonated the absorption maximum is at 568 nm, but when it is protonated it shifts to 603 nm. We followed this wavelength shift after a pH jump in stopped-flow experiments. Figure 1A shows absorbance traces for wild type BR after changing the pH from 2.8 to 6 and their fits to exponential kinetics. At 640 nm absorption decrease was observed; at 530 nm absorption rises; and near the isosbestic point of 585 nm nearly no change occurs, as expected from static titration (cf. below). About 75% of the amplitude of the absorbance decrease at 640 nm described a single exponential with a time-constant of 2.9 ms, while the rest occurred more slowly and its dependence on wavelength suggested that it originated from light-scattering changes. Purple membranes aggregate extensively at pH < 4, and this is reversed upon raising the pH evidently on the time scale of these measurements. This problem is minimized by prior dispersion of the membranes with sonication or by repeatedly passing the suspension through a low-bore syringe and/or using low salt concentration during the mixing. In most cases the absorbance traces from the pH jumps could be described adequately with a single exponential. The wavelength dependence of the signals was always checked to ascertain that they originated from the spectral change from the proton exchange reaction.

When the titration is in a homogeneous phase, the observed pseudo-first-order rate constants of the approach to a protonation/deprotonation equilibrium (Druckmann et al., 1995) will be linearly dependent on $[H^+]$. In BR that contained 13-(trifluoromethyl)retinal to lower its pK_a, proton exchange of the Schiff base in the unphotolyzed chromophore was indeed pH dependent in this way, although the absence of this dependence at pH < 6 suggested that when the rate of proton capture was high enough another reaction began to limit (Druckmann et al., 1995). Protonation of the retinal Schiff base in the photocycle of the D96N mutant (Otto et al., 1989; Holz et al., 1989; Miller & Oesterhelt, 1990; Cao et al., 1991), particularly in the presence of the weak acid azide (Tittor et al., 1989, 1994; Brown & Lanyi, 1996), also

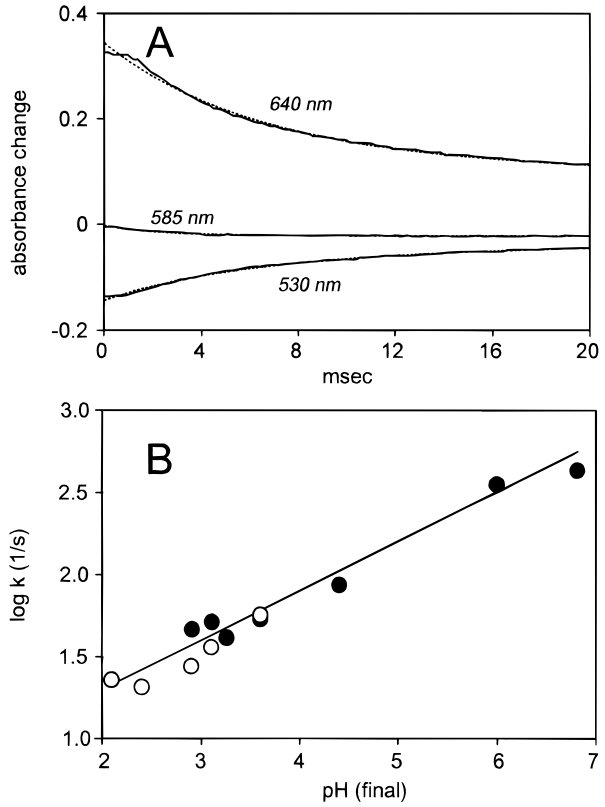

FIGURE 1: Kinetics and rate constants of absorbance changes in wild type BR after pH jump. (A) Time course of absorption change at 640, 585, and 530 nm after the pH was shifted from 2.8 to 6 in stopped-flow experiments. Solid lines, absorbance traces; dashed lines, exponential fits as explained in the text. (B) Pseudo-first-order rate constants as functions of the final pH after pH jump. Open circles, downshift of pH from an initial pH 7.0; closed circles, upshift of pH from 2.8 to indicated final pH. Conditions as described in Materials and Methods.

behaved in this way. We followed absorption changes in experiments similar to those in Figure 1A, but with pH jumps in both directions in order to obtain such information for the protonation reactions of Asp-85. Figure 1B shows the observed pseudo-first-order rate constants as functions of the final pH. The rate constants of about 30 and 400 s^{-1} for protonation and deprotonation near pH 2.5 and 7, respectively, are consistent with the recently reported single values of 30 and 184 s^{-1} under similar conditions (Druckmann et al., 1995). The rate constants for protonation and deprotonation (open and closed circles, respectively) *increased* weakly (with a slope of 0.3 for log *k* vs pH), rather than decreased (with a slope of unity), with pH. Proton exchange of Asp-85 with the aqueous medium must be therefore determined by an internal proton transfer step, which has some degree of pH dependence (cf. below).

Figure 2 shows the amplitudes of absorbance changes at various wavelengths in the stopped-flow measurements. The amplitude spectrum agrees well with the difference spectrum obtained in a static titration of dark-adapted BR (line).

Relationship of the Deprotonation of Asp-85 and the Decay of the O Intermediate. Stopped-flow pH jump experiments, as in Figure 1A with wild type, were performed with various BR mutants in which the decay of the O state is slower than in the wild type. Comparison of the rate of the deprotonation of Asp-85 with the rate of the decay of the O intermediate, determined by following absorbance change at 640 nm after flash photoexcitation (Cao et al., 1993), would test whether

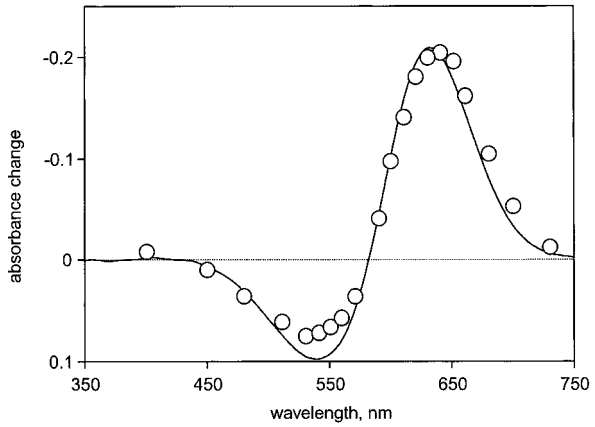

FIGURE 2: Amplitude spectrum for the absorbance changes detected after pH jump in wild type BR. Circles, absorbance change as a function of wavelength after raising the pH from 2.8 to 7.6; line, scaled difference spectrum from before and after raising the pH of a dark-adapted BR sample from 2.8 to 7.6 in a static experiment.

in these proteins the proton movement from Asp-85 toward the extracellular surface is what determines the rate of the last photocycle reaction. In the first class of mutants tested the protonation pathway between Asp-85 and the extracellular surface was perturbed. E204D and E204Q are representative of this group (Brown et al., 1995). In the second class of mutants the retinal binding pocket was perturbed. Mutations at Leu-93 (Subramaniam et al., 1991; Cao et al., 1993; Tittor et al., 1994; Delaney et al., 1995; Delaney & Subramaniam, 1996), as well as Val-49 and Ala-53 (Greenhalgh et al., 1993a; Brown et al., 1994a), belong in this group, although whether the red-shifted state should be called O or N is not clear (cf. below). Figure 3, parts A and B, shows the relationship of the rate constants from the pH jump experiments and from the photocycle measurements (all determined at pH 7).

For the mutants of the first class, shown in Figure 3A, the decay of O is somewhat slower than the deprotonation. This is in contrast to the nearly simultaneous deprotonation of Asp-85 in the photocycle and the decay of O.[2] The difference may reflect structural differences between the O intermediate and the acid blue form of BR, e.g., the presence of a twisted retinal chain and perturbations of the peptide bonds in the O intermediate. In spite of this, the two kinds of rate constants are well correlated over about 3 orders of magnitude. Moreover, the effect of D_2O substitution for the E204Q mutant affected both rate constants, and by the unusually large factor of 10, suggesting a complex pathway for proton transfer. Also included in Figure 3A is Y185F/E204Q, because it has a particularly slow rate of O decay. The reason for the large additional photocycle perturbation in the double mutant is not clear, but Tyr-185 is located in the extracellular proton channel (Henderson et al., 1990), so we expected that the two rate constants would intersect on the line for the other Glu-204 mutants, and they do. For this class of mutants the decay of O is evidently related to the deprotonation of Asp-85. In contrast, for the mutants of the second class, shown in Figure 3B, the two kinds of rate constants are not at all correlated. Among these mutants the decay of O varies from that in wild type by about 2.5 orders of magnitude, while the deprotonation is nearly constant. As expected, D_2O substitution slows the deprotonation of Asp-85 in L93T by about 10-fold, but does not affect the decay of the O intermediate. In this class of

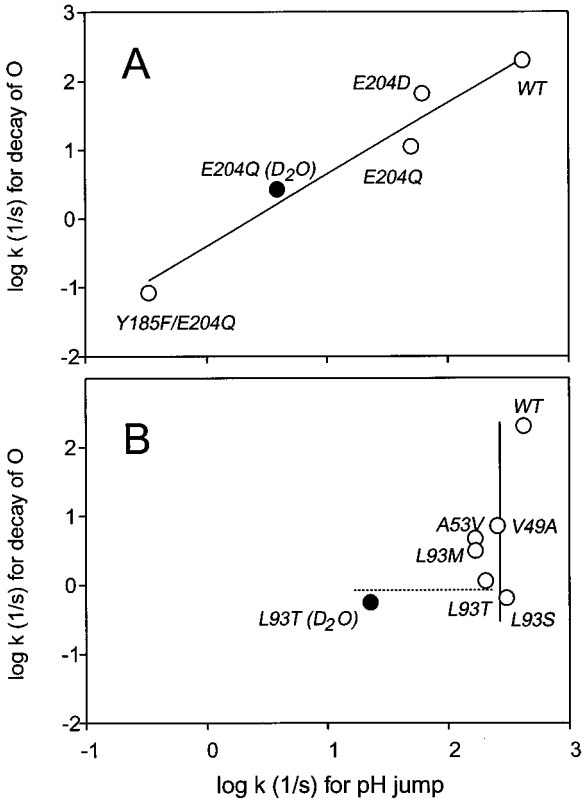

FIGURE 3: Correlation of the pseudo-first-order rate constants for the decay of the O state in the photocycle (ordinate) and the deprotonation of Asp-85 in pH jump experiments (abscissa). The O decays were measured at pH 7, and the pH jumps were from pH 3 to 7. The points refer to either the wild type protein (WT) or the mutants as indicated next to the symbols. Closed circles: data from D_2O.

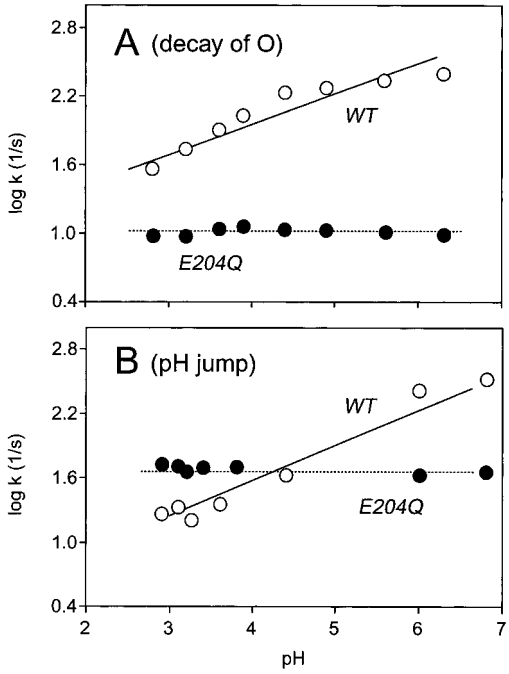

FIGURE 4: Dependence of the pseudo-first-order rate constants for the decay of the O state in the photocycle (A) and the deprotonation of Asp-85 in pH jump experiments (B) on pH. Open circles, wild type protein; closed circles, E204Q mutant.

mutants the lifetime of the last photointermediate is longer for reasons not related to deprotonation of Asp-85.

Another kind of correlation between the rate constants for the decay of the O photointermediate in the first class of mutants and the deprotonation of Asp-85 in pH jump experiments is evident in their pH dependencies. Figure 4A shows the rate constants for O decay in the wild type and the E204Q mutant as functions of pH. The rate constant increases with pH in the wild type but not in the mutant. The reason for this is not yet clear. Evidently, Glu-204 confers a degree of pH dependence onto the rate-limiting step in the decay of the O state. The data in Figure 4B indicate that this is true also for the deprotonation of Asp-85 in the unphotolyzed protein (see also Figure 1B for data with the wild type). The deprotonation upon raising the pH from 2.8 to the values indicated is somewhat more rapid than the O decay in the E204Q mutant, as in Figure 3A, but the pH dependence of the two rate constants observed in the wild type is absent in the mutant. Although the data are not shown, the two kinds of rate constants measured for E204D were found to be also virtually pH independent. Thus, in the wild type protein both the O decay and the deprotonation of Asp-85 depend on a surface process that involves the local environment of the carboxylic group of Glu-204. It may be the negative electrical potential of the extracellular surface, since the ionization of numerous and varied surface groups increases it roughly linearly with pH (Jonas et al., 1990; Alexiev et al., 1994; Cao et al., 1995).

DISCUSSION

The BR photocycle consists of the sequential thermal reactions of the chromophore and the protein that describe the pathway leading to the reisomerization of the photoisomerized 13-*cis*-15-*anti*-retinal. It might be expected that the interconversions of the various unstable intermediate states that arise in this process would have little to do with the initial stable state. It has become evident in recent years, however, that some reactions of the photocycle can be understood by finding and describing analogous reactions of the unphotolyzed protein. Thus, deprotonation of the retinal Schiff base of the unphotolyzed D85N mutant upon raising the pH from 7 to 11 produced a changed overall protein conformation similar to that observed in the M photointermediate, and the vectoriality of the proton exchange under these conditions was like that in the photocycle (Kataoka et al., 1994). Another example is the complex titration behavior of Asp-85 in the unphotolyzed protein that revealed the interaction of this residue with Glu-204 and suggested an elegant mechanism by which protonation of Asp-85 induces proton release to the extracellular surface in the photocycle and by raising the pK_a of Asp-85 determines the directionality of the transport (Balashov et al., 1995; Richter et al., 1996). In this report we show another property of the unphotolyzed protein that is relevant to a photocycle reaction. In some mutants the rate of the deprotonation of Asp-85, measured after raising the pH from 3 to 7 in the dark, is highly correlated with the rate of the decay of the O state in the photocycle. These rates are influenced similarly by the E204Q mutation that interferes with the passage of protons in the extracellular half-channel that connects Asp-85 and the protein surface (Brown et al., 1995), by D/H isotope exchange, and by pH. Under these conditions, therefore, it appears that deprotonation of Asp-85 determines the rate of decay of the O state. This should not be surprising, in view of the fact that it is during the

decay of O that Asp-85 loses the proton it had acquired in the M state, either directly to the aqueous medium at pH < 5−6 (Zimányi et al., 1992) or to Glu-204 at higher pH (Brown et al., 1995).

In another class of mutants, such as those of Leu-93, Ala-53, and Val-49, residues that contact the retinal chain, we find that a correlation between the rates of the deprotonation of Asp-85, measured with pH jump, and the decay of O does not exist. In these cases it is another reaction that determines the rate of O decay. Delaney et al. (1995) emphasized that Leu-93 is located near the 13-methyl group of the retinal and when replaced will interfere with the reisomerization of the retinal to *all-trans*. We confirmed that the red-shifted intermediate that accumulates contains 13-*cis*-retinal by measuring FTIR spectra of the photointermediates of various Leu-93 mutants.[2] These spectra also indicated that some aspects of the protein stucture in the photointermediate are more O-like than N-like. The results therefore raise the question of how the N and the O states should be defined. On the one hand, historically N was distinguished from O because it is blue-shifted relative to the unphotolyzed chromophore rather than red-shifted (Lozier et al., 1975). On the other, vibrational spectroscopy of these states has indicated that, in the wild type protein at least, N contains 13-*cis*-retinal (Fodor et al., 1988) while O contains *all-trans* (Smith et al., 1983). Since in mutants of Leu-93, such as L93M, the absorption maximum of the unphotolyzed chromophore is 20 nm blue-shifted and the difference maximum for the putative O state is farther to the blue than in wild type, it may be that in these proteins the N state is anomalous and red-shifted. This would occur if the perturbation at residue 93 were less for the 13-*cis* states than for the *all-trans* chromophore. A similar problem of nomenclature arose for the ambiguous N or O state of the D85E mutant (Lanyi et al., 1992). However, we do not consider such an ambiguity of nomenclature important. It does not preclude the conclusion that the rate-limiting step for the recovery of the initial state in these mutants must be the reisomerization of the 13-*cis*-retinal.

Thus, under some conditions deprotonation of Asp-85 and under others reisomerization of the $C_{13}-C_{14}$ double bond of the retinal limits the recovery of the initial state. How does the proton transfer relate to retinal bond motions? Clues for the causal sequence at the end of the photocycle are provided by the FTIR spectra of the E204Q and L93M (and related) mutants: (1) Since the long-lived O (or N) state that accumulates in L93M (because the 13-*cis* to *all-trans* isomerization is hindered) contains a protonated Asp-85 in spite of its O-like protein structure,[2] deprotonation of this residue must depend on the reisomerization step. (2) Since the long-lived O state that accumulates in E204Q (because deprotonation of Asp-85 is hindered, as shown by the slower deprotonation in the pH jump experiments) contains *all-trans*-retinal with HOOP bands,[2] relaxation of the twist of the retinal chain must depend on deprotonation of Asp-85. Figure 5 illustrates this sequence of events for the wild type protein and the steps affected by the two kinds of mutations. The mechanisms of the proposed causal relationships in this sequence are uncertain, but they are likely to originate from the dependence of the barriers for various bond rotations in the retinal on the charge environment of the Schiff base (Tavan et al., 1985) on the one hand, and the dependence of the pK_a of the Schiff base and therefore the deprotonation

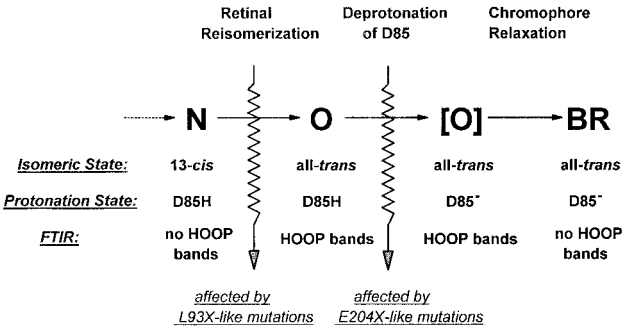

FIGURE 5: Schematic representation of the events at the end of the BR photocycle. Reisomerization of the retinal to *all-trans* triggers deprotonation of Asp-85, and the ensuing deprotonation of Asp-85 triggers relaxation of a twist in the retinal chain that gives rise to HOOP bands in FTIR spectra (cf. text). The species [O] is postulated to exist but has not been detected. Mutations at residue 93 interfere with reisomerization of the retinal to *all-trans* (Delaney et al., 1995; Kandori et al., 1996b), although not with some of the protein structural changes that occur in the N to O reaction [not shown in this scheme, footnote 2]. Mutations at residue 204 interfere with deprotonation of Asp-85 (cf. text).

reaction of Asp-85 on the electron distribution along the retinal chain (Schulten & Tavan, 1978; Orlandi & Schulten, 1979) on the other. The distance and/or orientation of the Schiff base relative to Asp-85 will also affect the pK_a of Asp-85 (Brown et al., 1994a), and this will be changed when the Schiff base is displaced upon reisomerization of the retinal. The dependence of the relaxation of the twist in the retinal chain on deprotonation of Asp-85 predicts that there should be a transient O state (designated as [O] in Figure 5) with deprotonated Asp-85 but high-amplitude HOOP bands. Such an intermediate has not been detected, but its existence seems reasonable on the grounds that each physical event in the photocycle is, in principle, a separate step even when it merges kinetically with others.

The involvement of the deprotonation of Asp-85 in the decay of the O intermediate suggests that the reason for the irreversibility of the recovery of the initial state is in the ΔpK_a that governs the last proton transfer. The great difference between the pK_a of Glu-204 and the pK_a of Asp-85 after reisomerization of the retinal will cause extensive dissipation of free energy. This is as expected if the pK_a values of Asp-85 and Glu-204 assume their values in the unphotolyzed protein at this time, i.e., 2.5 and 9.2, respectively (Chang et al., 1985; Richter et al., 1996). A somewhat different situation occurs in E204Q, and in the wild type protein at a pH where Glu-204 does not deprotonate. In these cases Asp-85 loses its protein directly to the bulk, and it is the difference between the pH and the pK_a of Asp-85 that determines whether the last step in the photocycle is irreversible.

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