# A Linkage of the p$K_a$'s of asp-85 and glu-204 Forms Part of the Reprotonation Switch of Bacteriorhodopsin[†]

Hans-Thomas Richter,[‡] Leonid S. Brown,[‡] Richard Needleman,[§] and Janos K. Lanyi*,[‡]

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

*Received December 6, 1995; Revised Manuscript Received January 24, 1996*[⊗]

ABSTRACT: Because asp-85 is the acceptor of the retinal Schiff base proton during light-driven proton transport by bacteriorhodopsin, modulation of its p$K_a$ in the photocycle is to be expected. The complex titration of asp-85 in the unphotolyzed protein was suggested [Balashov, S. P., Govindjee, R., Imasheva, E. S., Misra, S., Ebrey, T. G., Feng, Y., Crouch, R. K., & Menick, D. R (1995) *Biochemistry 34,* 8820−8834] to reflect the dependence of this residue on the protonation state of another, unidentified group. From the pH dependencies of the rate constant for the thermal equilibration of retinal isomeric states (dark adaptation) and the deprotonation kinetics of the Schiff base during the photocycle in the E204Q and E204D mutants, we identify the residue as glu-204. The nature of its interaction with asp-85 is that at neutral pH either residue can be anionic but not both. This is consistent with our recent finding that glu-204 is the origin of the proton released to the extracellular surface upon protonation of asp-85 during the transport. We propose, therefore, that the following series of events occur in the photocycle. Protonation of asp-85 in the proton equilibrium with the Schiff base of the photoisomerized retinal results in the dissociation of glu-204 and proton release to the extracellular surface. The deprotonation of glu-204, in turn, raises the p$K_a$ of asp-85, and the equilibrium with the Schiff base shifts toward complete proton transfer. This constitutes the first phase of the reprotonation switch because it excludes asp-85 as a donor in the reprotonation of the Schiff base that follows. The sequential structural changes of the protein that ensue, detected earlier by diffraction, are suggested to facilitate the change of the access of the Schiff base toward the cytoplasmic side as the second phase of the switch, and the lowering the p$K_a$ of asp-96, so as to make it a proton donor, as the third phase.

It is widely assumed that ion translocation by active transport systems is based on the energy-driven cycling of the protein through two conformations in which the access of a single binding site alternates between the two membrane surfaces [reviewed recently in Lanyi (1995)]. In the light-driven proton pump, bacteriorhodopsin, this binding site is the protonated retinal Schiff base (for reviews, see Oesterhelt et al. (1992), Rothschild (1992), Lanyi (1993), and Ebrey (1993)]. The first transport step after photoisomerization of the retinal from *all-trans* to 13-*cis* is the transfer of the proton from the Schiff base to the anionic asp-85, which has access to the extracellular surface (the L → $M_1$ reaction of the photochemical cycle).[1] The Schiff base is then reprotonated by the initially protonated asp-96, which has access to the cytoplasmic surface (the $M_2$ → N reaction). The change of the connectivity of the Schiff base from proton donor at one membrane side to proton acceptor at the other (the intervening $M_1$ → $M_2$ reaction) constitutes the alternating access in this transport cycle (Nagle & Mille, 1981; Schulten et al., 1984; Fodor et al., 1988; Henderson et al., 1990; Váró & Lanyi, 1991a; Kataoka et al., 1994) and was termed the *reprotonation switch*. Little has been known about its molecular mechanism. In principle, as in any ion pump, there are two possibilities (Kalisky et al., 1981; Lanyi, 1993). In an "affinity" mechanism proton transfer pathways exist in both directions throughout, but the p$K_a$ of asp-85 becomes high after its protonation while the p$K_a$ of asp-96 is lowered relative to the p$K_a$ of the Schiff base, so in the next step asp-96 rather than asp-85 is the proton donor. In an "accessibility" mechanism the p$K_a$'s would allow unproductive proton transfers, but the geometry of the three groups is so regulated that the unprotonated Schiff base loses its access to the protonated asp-85 and becomes connected to asp-96 instead. The reprotonation switch might be based on either of these mechanisms or contain elements from both.

Questions concerning these mechanistic alternatives can be asked more specifically by considering the structure of the protein. According to the structural model (Henderson et al., 1990) based on cryoelectron diffraction of the crystalline two-dimensional lattice of the purple membrane, most of the 248 residues are in seven transmembrane helices, A through G. The retinal chain is at a small angle to the plane of the membrane, with the Schiff base, bound to lys-216 on helix G, located in the interhelical cavity at about 20 Å from the extracellular surface. Proton conduction pathways, "half-channels", extend from the Schiff base to both

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

* Author to whom correspondence should be addressed.

[‡] University of California.

[§] Wayne State University.

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: L, M, N, and O refer to some of the photointermediates of bacteriorhodopsin, while BR is the unphotolyzed state; bis-tris-propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

0006-2960/96/0435-4054$12.00/0 

membrane surfaces. The counterion to the positively charged Schiff base is a diffuse hydrogen-bonded network in the extracellular half-channel that contains asp-85, asp-212, arg-82, and hydrogen-bonded water (de Groot et al., 1989, 1990; Dér et al., 1991). The events that take place in this region link the proton transfer to asp-85 in the photocycle to the release of a proton to the extracellular surface. The source of the released proton was recently identified as glu-204, located near the surface (Brown et al., 1995a). A more detailed structural model based on molecular dynamics calculations (Humphrey et al., 1994) places two hydrogen-bonded water molecules between the Schiff base and asp-85, locates arg-82 within hydrogen-bonding distance from asp-85, and connects arg-82 to glu-204 via three hydrogen-bonded water molecules.

It should be expected that in this complex extracellular region all functional groups will affect one another. Indeed, close interaction of asp-85 and arg-82 was inferred from the greatly raised p$K_a$ of asp-85 in R82A and Q mutants (Subramaniam et al., 1990; Otto et al., 1990; Thorgeirsson et al., 1991; Brown et al., 1993; Balashov et al., 1993), and the fact that the proton release to the surface is much delayed in these mutants relative to the protonation of asp-85 (Balashov et al., 1993; Brown et al., 1994; Cao et al., 1995). Replacement of both asp-212 and arg-82 with neutral residues appeared to raise the p$K_a$ of the Schiff base (Brown et al., 1995b) while replacement of asp-85 lowered it (Marti et al., 1991b; Turner et al., 1993; Tittor et al., 1994; Kataoka et al., 1994). The hydrogen-bonded state of bound water changes in the photocycle, and this change is affected by mutation of each of these residues (Maeda et al., 1992, 1994; Kandori et al., 1995). Significantly, titration of asp-85 in the wild type protein as well as in the R82A and R82K mutants has indicated that the p$K_a$ of asp-85 is reciprocally linked to the p$K_a$ of an unidentified group in this region (Balashov et al., 1993, 1995, 1996). Arg-82 is either this residue or has a strong effect on this linkage.

What are the relative p$K_a$'s of the proton donor Schiff base and acceptor asp-85 during the L $\rightarrow$ $M_1$ and the $M_2$ $\rightarrow$ N reactions of the photocycle, *i.e.*, before and after the reprotonation switch? Kinetic analyses of the former reaction suggested that, although in the unphotolyzed protein the two p$K_a$'s are at least 5 pH units apart (Brown et al., 1993), the p$K_a$ difference is not far from zero during the proton transfer in the L $\rightarrow$ $M_1$ reaction (Váró & Lanyi, 1991a; Zimányi et al., 1992b). There is evidence, however, that in the ensuing steps the two p$K_a$'s diverge once again. The p$K_a$ of the Schiff base after protonation of asp-85 in the photocycle could be determined directly in the D96N mutant (Brown & Lanyi, 1996). It is 8.3 and probably not very different in the wild type protein. Observation of the characteristic COOH vibrational band of asp-85 in the long-lived M and N intermediates above pH 10 and a recent interpretation of its unusually high frequency suggested, however, that at this time in the photocycle the p$K_a$ of this residue is >10, and for the reason that there is a large decrease in the local dielectric constant (Braiman et al., 1996). Is the protonation equilibrium between the Schiff base and asp-85 established in the L $\leftrightarrow$ $M_1$ reaction shifted during the $M_1$ $\rightarrow$ $M_2$ reaction toward virtually complete proton transfer because the p$K_a$ of asp-85 becomes much higher than 8 (as part of an affinity switch), and, if so, what molecular mechanism would cause the suggested change in the dielectric environment of asp-85?

In this report we concentrate on these initial events in the reprotonation switch. We have explored the possibility of a mechanism suggested by the anomalous behavior of asp-85 (Balashov et al., 1993, 1995, 1996), by which the p$K_a$ of asp-85 might be regulated during the photocycle in the way required for an affinity switch. Titrations of the dark adaptation of wild type, E204Q, and E204D proteins, and kinetic description of the pH dependence of the rise of the M state in their photocycles demonstrate that the protonation states of asp-85, arg-82, and glu-204 are linked together in the way suggested for asp-85 and the postulated protonatable group (Balashov et al., 1995, 1996). It appears, therefore, that the unknown group is glu-204, linked to asp-85 through arg-82 in a hydrogen-bonded network of water molecules as in a recently calculated energy-minimized structure (Humphrey et al. 1994). Thus, protonation of asp-85 in the photocycle will cause the lowering of the p$K_a$ of glu-204, and the ensuing loss of its proton to the extracellular medium will raise the p$K_a$ of asp-85. From this mechanism, and what can be deduced for the events that immediately follow it, we construct a hypothesis for the rest of the events that must comprise the reprotonation switch. Remarkably, bound water seems to play a more active role in causing changes of the p$K_a$'s along the proton transport trajectory than previously suspected.

## MATERIALS AND METHODS

Purple membranes were prepared from *Halobacterium salinarium* by a standard method (Oesterhelt & Stoeckenius, 1974). The E204Q and E204D mutants used have been described before (Brown et al., 1995a). Dark adaptation was followed at 590 nm in a Shimadzu model 1601 spectrophotometer connected to a desktop computer. Absorption changes were followed after photoexcitation with an Nd: Yag laser pulse (532 nm, 7 ns), as in earlier publications of ours [*e.g.*, Váró et al. (1995)]. The temperature was 22 °C throughout.

## RESULTS

*pH Dependence of the Rate of Dark Adaptation in Wild Type Bacteriorhodopsin and the R82Q, E204Q, and E204D Mutants*. In the pH region where the fraction of protonated asp-85 is greater than a few percent, asp-85 can be titrated in unphotolyzed bacteriorhodopsin on the basis of a red-shift of the absorption maximum when this residue is protonated. At pH much higher than the p$K_a$ detected in this way, at about 2.5, the amount of red-shifted species is difficult to determine by such spectroscopic titration. However, Balashov et al. (1995, 1996) have shown that the rate of the thermal equilibration of the retinal isomeric states (dark adaptation) is directly proportional to the fraction of asp-85 that is protonated, and this constitutes a method of titrating asp-85 with a far greater dynamic range. Wild type bacteriorhodopsin was light adapted at various pH so as to accumulate *all-trans* retinal and incubated in the dark while following absorption at 590 nm to determine the rate of isomeric re-equilibration. Figure 1 shows examples of such traces. In every case at pH > 3 they could be satisfactorily fitted with a single exponential (dotted lines). At lower pH small deviations from such simple kinetics occurred, probably

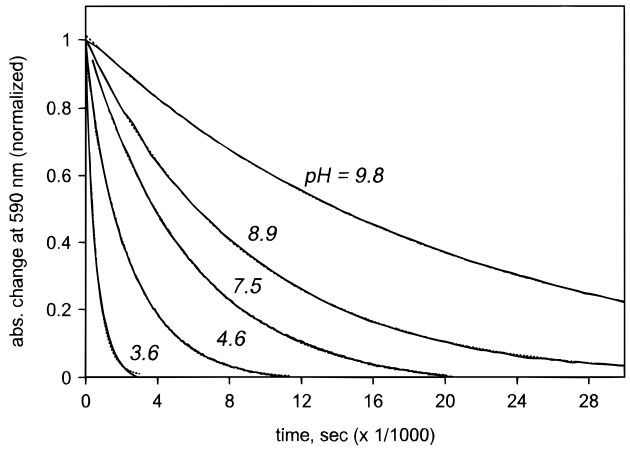


FIGURE 1: Absorbance change at 590 nm during dark adaptation of wild type bacteriorhodopsin. The measured traces at the indicated pH (solid lines) are shown together with the best fits to single exponentials (dotted lines). Conditions: 15 $\mu$M bacteriorhodopsin, 100 mM NaCl, 40 mM phosphate, 20 mM bis-tris propane, at the pH indicated. Light adaptation before the experiments, 2−10 min with white light.

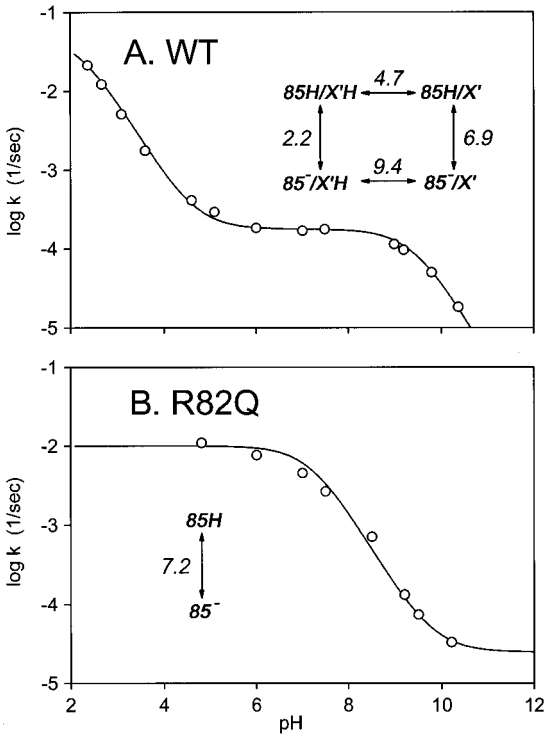


FIGURE 2: pH dependence of the rate of dark adaptation in wild type (A) and R82Q (B) bacteriorhodopsins. The data are from experiments such as in Figure 1. The lines represent the best fits of the schemes shown as inserts, with the p$K_a$ values given. The multiphasic titration curve is explained with the interaction of asp-85 with an unknown group, X′H (Balashov et al., 1995). In the wild type the two apparent p$K_a$'s and the amplitude of the plateau between them define three of the microscopic p$K_a$'s. The fourth p$K_a$ is determined by the other three. In R82Q the data do not implicate a second group.

caused either by a multiplicity of chromophore states (Mowery et al., 1979; Váró & Lanyi, 1989) or by additional photoreactions (Balashov et al., 1996), but we ignored this complication in spite of the fact that the calculated p$K_a$ was slightly shifted to lower values as a result.

If dark adaptation is catalyzed by the protonation of asp-85 (Balashov et al., 1995), then the isomeric equilibration will follow the protonation equilibration of asp-85. However, on the basis of time-resolved titrations of asp-85 which demonstrated that this process is on the millisecond time scale (Druckmann et al., 1985, 1995), it can be assumed that the rates measured are not limited by this protonation equilibration but by the isomerization step. The rates of dark adaptation at different pH values will be therefore proportional to the fractional concentration of protonated asp-85. At pH < 4 the amplitudes of the absorption changes became lower, mostly because when asp-85 is protonated the light-adapted mixture contains 13-*cis* retinal (de Groot et al., 1990). Nevertheless, the relationship between the rate of measured dark adaptation and the protonation state of asp-85 should hold.

Figure 2 shows the first-order rate constants of dark adaptation, measured as in Figure 1, for wild type (panel A) and the R82Q mutant (panel B). The data reproduce well the results of earlier studies of the wild type protein and the R82A mutant (Ohno et al., 1977; Balashov et al., 1993). The lower apparent p$K_a$ in the wild type is about the same as that detected from the color shift upon deprotonation of the bulk of asp-85 (Chang et al., 1985; Subramaniam et al., 1990, 1992; Metz et al., 1992; Jonas & Ebrey, 1991). The explanation given for the higher p$K_a$ (Balashov et al., 1995, 1996) was that interaction with a second protonatable group X′H influences the observed titration behavior of asp-85 according to the scheme shown as the inset to Figure 2A. The results with R82Q (Figure 2B), in which the single elevated p$K_a$ observed corresponds to that measured from the color shift under these conditions, as also observed earlier with R82A (Balashov et al., 1995), suggest either that X′H is arg-82 or that arg-82 strongly perturbs its interaction with asp-85. Because the rate at high pH seemed to reach a limiting value a basal rate, that was several orders of magnitude lower than with protonated asp-85, was added to the scheme to fit the data. The implication of the scheme for the wild type protein (Balashov et al., 1993, 1996; Figure 2A) is that when X′H is protonated the p$K_a$ of asp-85 is 2.2, but when it is deprotonated this p$K_a$ is increased to 6.9. Conversely, when asp-85 is unprotonated, the p$K_a$ of X′H is 9.4, but when it is protonated this p$K_a$ is lowered to 4.7.

Since glu-204 was identified as the group that releases a proton to the extracellular surface upon protonation of asp-85 in the photocycle (Brown et al., 1995a), this residue is a candidate for X′H. Figure 3 shows the pH dependence of the rate of dark adaptation for E204D (panel A) and E204Q (panel B). If glu-204 is X′H, then its replacement with a neutral residue will eliminate the higher p$K_a$. Indeed, the dark adaptation of E204Q is described with a single p$K_a$ that corresponds to that measured for the color shift upon deprotonation of asp-85 (not shown). The results with the E204D mutant reveal two p$K_a$'s, as expected from this conservative residue replacement. In this mutant the p$K_a$ of X′H is lowered by about 1 pH unit, with asp-85 both protonated and unprotonated, which is also as expected from the generally lower aspartate p$K_a$. Thus, like arg-82, glu-204 is functionally equivalent to the postulated X′H. However, unlike the large perturbation in the arg-82 mutants, its replacement in E204Q eliminates the higher p$K_a$ without affecting the lower one, as expected from simply eliminating the possibility that residue 204 can become anionic. We will argue (cf. below) that this strongly suggests that X′H is glu-204 rather than arg-82.

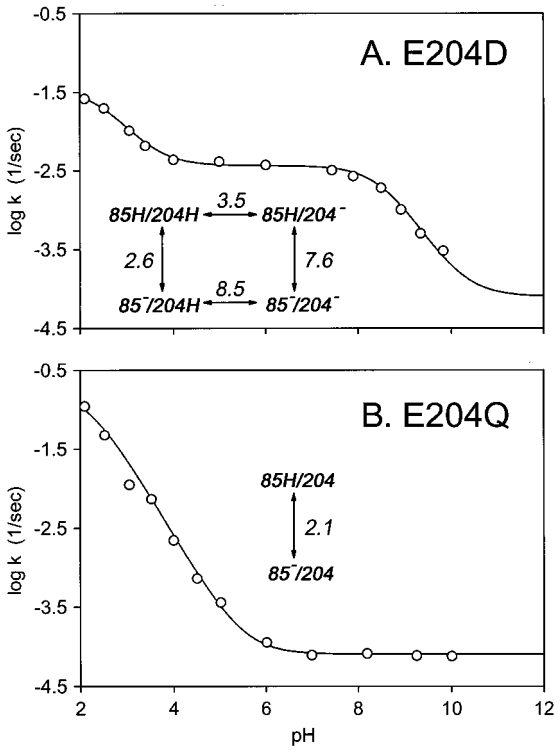


FIGURE 3: pH dependence of the rate of dark adaptation in E204D (A) and E204Q (B) bacteriorhodopsins. The data are from experiments such as in Figure 1. As in Figure 2, the lines represent the best fits of the schemes shown as inserts, with the p$K_a$ values given. The arguments in the text identify group X′H in Figure 2A as glu-204.

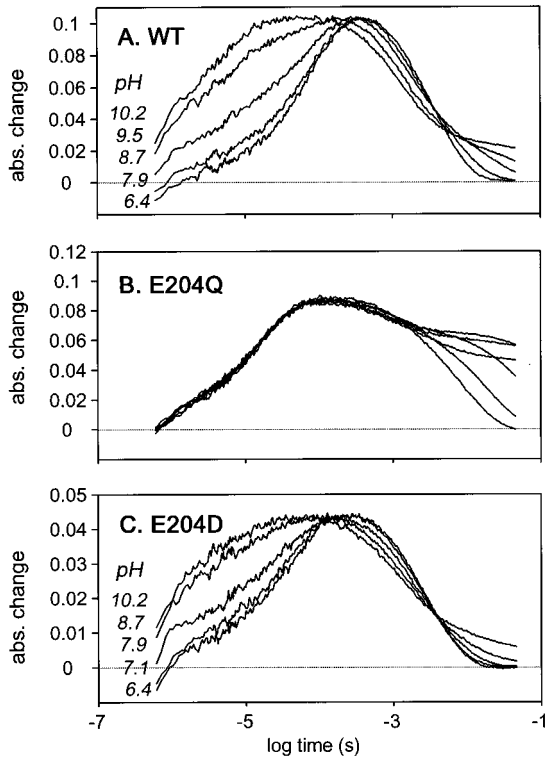


FIGURE 4: Kinetics of the M state after flash excitation of wild type bacteriorhodopsin (A), and the E204Q (B) and E204D (C) mutants. Absorption change at 410 nm is plotted. Conditions: 15 $\mu$M bacteriorhodopsin, 100 mM NaCl, 12.5 mM bis-tris propane, 12.5 mM CAPS at the pH indicated.

*pH Dependence of the Rise Kinetics of the M Intermediate in the Photocycles of Wild Type Bacteriorhodopsin and the E204Q and E204D Mutants*. The photocycle events that result in deprotonation of the retinal Schiff base have been described near neutral pH (Zimányi et al., 1992b) with the sequence

$$K \leftrightarrow L \leftrightarrow M_1 \rightarrow M_2$$

where each of the three reactions contributes a kinetic component to the rise of what is detected spectroscopically as the M state. Thus, both equilibration reactions will be influenced by changes in the p$K_a$ of asp-85. The rise of this p$K_a$ induced by its coupling to the proton release group will occur only during the subsequent $M_1 \rightarrow M_2$ reaction where the proton is released (Zimányi et al., 1992b) and would not be relevant to the $K \leftrightarrow L \leftrightarrow M_1$ equilibrium. However, any change that originates from deprotonation of this group *before* the photoreaction should have an effect. Indeed, it has long been known (Liu, 1990; Váró & Lanyi, 1990; Bitting et al., 1990; Balashov et al., 1993) that the overall formation of M is greatly accelerated at pH > 8. This acceleration was identified as an apparent one, which instead reflects the increase of the amplitude of the more rapid rise components (Balashov et al., 1991, 1995; Cao et al., 1995). The p$K_a$ defined by this effect on the M rise, with a value of 9.2 (Balashov et al., 1991), has been attributed to a tyrosine or possibly arg-82, and indeed, the rate of the formation of M in the R82A mutant is rapid and pH independent (Balashov et al., 1993).

Figure 4 shows absorption changes at 410 nm after photoexcitation of wild type bacteriorhodopsin and of the E204Q and E204D mutants. It is evident that large changes of the rise of the M state occur in the pH 6−10 range in the wild type and E204D but not in E204Q. The change is the shift of the bulk of the rise from the slowest component to the more rapid one(s). The magnitude of this effect could be most conveniently calculated by fitting the traces to three rise and two decay components (not shown). The sum of the amplitudes of the three rise components corresponds to the total concentration of the deprotonated intermediate. The sum of the concentrations of the states that remain protonated after the $K \leftrightarrow L \leftrightarrow M_1$ equilibrium is established was therefore given by the amplitude of the slowest of the three rise components. This amplitude, scaled so that its limiting value at low pH is 1, is given as a function of pH in Figure 5. Balashov et al. (1991) had calculated 1 *minus* the amplitude of the slowest rise component with similar logic. The apparent p$K_a$ in Figure 5 is 9.2 for the wild type, as reported before, but is 8.3 for E204D. The two values correspond reasonably well to the predicted p$K_a$'s for residue 204 when asp-85 is anionic, *i.e.*, in the unphotolyzed state (Figures 2A and 3A). Because the p$K_a$'s of asp-85 and glu-204 are linked, at higher pH where glu-204 is dissociated asp-85 enters the photocycle with a raised p$K_a$. This will shift the protonation equilibria $K \leftrightarrow L \leftrightarrow M_1$ toward increased amounts of M. As expected, there is no effect of pH on this equilibrium in E204Q.

## DISCUSSION

The nature of the reprotonation switch continues to be the most puzzling part of the bacteriorhodopsin photocycle. Understanding it at a molecular level requires that the relevant residue interactions near the Schiff base be dissected

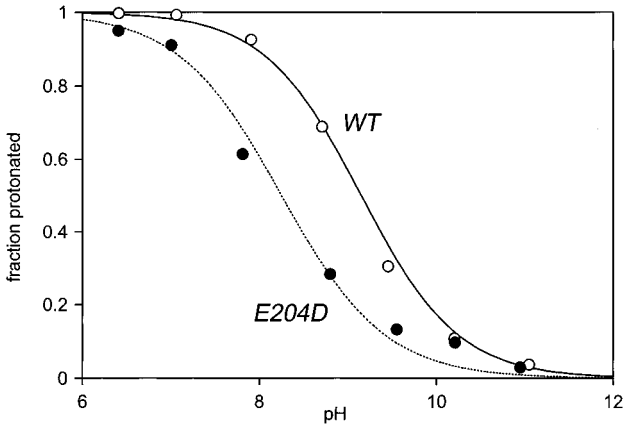


FIGURE 5: pH dependence of the amplitude of the last phase of the formation of the M intermediate in the wild type (open circles) and E204D (closed circles). The amplitudes are calculated from the data from Figure 4 and are given as the fraction of the Schiff base still protonated before this step relative to its limiting value at low pH.

from the large number of complex changes observed when functional residues in this region are mutated. An important step toward this goal was the identification of a specific interaction of asp-85 with another protonatable residue, X′H (Balashov et al., 1995, 1996). Its influence on the p$K_a$ of asp-85 and *vice versa* made this residue a key to the proton transport mechanism. In this report we present evidence that X′H is glu-204 and propose a mechanism for its participation in the reprotonation switch. If correct, then this mechanism also defines the likely roles of the protein structural changes that follow it in this process.

*Linkage between the p$K_a$'s of asp-85 and glu-204.* The interconversions of the four possible states of the asp-85−X′H system in the model of Balashov et al. (1995, 1996) are described by four p$K_a$'s, and their interaction results in two observed apparent p$K_a$'s for the titration of asp-85. In Figure 2 we confirm this behavior for the wild type protein and its perturbation in the R82Q mutant. In Figure 3 we demonstrate that the second p$K_a$ is lowered by about one pH unit in E204D and is missing entirely in the E204Q mutant. The pH dependence of the rise kinetics of the M intermediate likewise identifies a protonatable residue with influence on the p$K_a$ of asp-85 in its protonation equilibrium with the Schiff base. The pH independence of this process in R82A and R82K (Balashov et al., 1993, 1995) suggested that the residue might be arg-82, but the results with the E204Q and E204D mutants (Figures 4 and 5) demonstrate that glu-204 is also a candidate. In principle, therefore, either arg-82 or glu-204 (or a third unsuspected residue affected by them, or even bound water) could be X′H. Balashov et al. (1995) suggested that X′H might be arg-82 because the p$K_a$ of X′H in the R82K mutant was lowered by 1.7 pH units, consistent with the lower p$K_a$ of a lysine. Similar arguments can now be made for glu-204, from its replacement with glutamine which abolishes the effects of X′H, or with aspartate, which lower its p$K_a$. However, the p$K_a$ of a carboxylic acid is inherently lower than that of a guanidinium group, and would be in the right pH range for what is calculated for X′H. Further, the appearance of a new negative C═O stretch band at 1714 cm$^{-1}$ in the M state of E204D suggested that it is residue 204 that deprotonates upon protonation of asp-85 in the photocycle (Brown et al., 1995a). According to these arguments X′H is glu-204. Nevertheless,

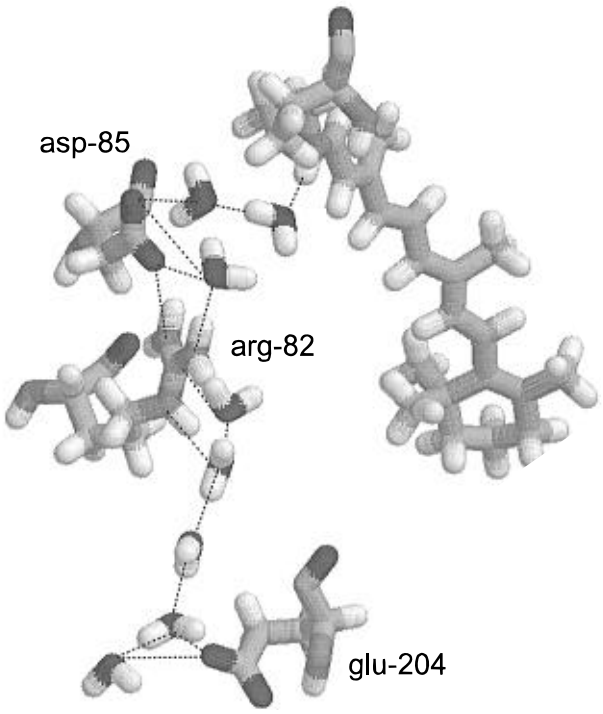


FIGURE 6: Energy-minimized structure of the extracellular region of bacteriorhodopsin [from Humphrey et al. (1994)] showing the retinal Schiff base (right-hand side), asp-85, arg-82, glu-204, and eight of the 15 proposed internal water molecules. Possible hydrogen bonds are shown as dotted lines.

the participation of arg-82 in X′H cannot be entirely excluded, particularly since the amplitude of the negative C═O stretch band that originated from deprotonation of asp-204 was less than expected, and the possibility of some degree of delocalization of the proton was raised (Brown et al., 1995a). Since recent results (Dioumaev & Braiman, 1995) indicate that there is no inherent reason why the C═O stretch frequency of an aspartatic acid should be more than 1−2 cm$^{-1}$ higher than that of a glutamic acid in the same environment, the appearance of this band in E204D suggests that the aspartate interacts differently than glutamate with the groups around residue 204. For this reason, also, it is possible that in the wild type protein arg-82 has a greater role in proton release than in the E204D mutant. However, for the sake of simplicity we suggest the scheme given in Figure 3A, which replaces X′H explicitly with glu-204. Thus, when residue 204 is replaced with glutamine, 85H/204$^-$ and 85$^-$/204$^-$ cannot form, and therefore the simple consequence is that the first p$K_a$ for asp-85 is unchanged but the second p$K_a$ is eliminated (Figure 3B).

The scheme predicts that at low pH both asp-85 and glu-204 are protonated, but as the pH is raised a mixture of states will be produced in which either asp-85 or glu-204 (but not both) is deprotonated. Importantly, when the pH is raised again, the p$K_a$'s of the two further deprotonation reactions that lead to the fully unprotonated asp-85/glu-204 pair are greatly changed, indicating that the protonation states of asp-85 and glu-204 are dependent on one another. A structural basis for such an interaction is provided by a model derived from molecular dynamics, which placed a hydrogen-bonded water between asp-85 and arg-82 and a chain of three hydrogen-bonded water molecules between arg-82 and glu-204 (Humphrey et al., 1994). This is illustrated in Figure 6, which shows the positions of the relevant residues, retinal

Schiff base, asp-85, arg-82, and glu-204, and the water molecules in this region of the structural model. Calculation of the p$K_a$'s from an electrostatic model (Scharnagl et al., 1995) also connected these residues functionally and predicted that glu-204 will dissociate once asp-85 is protonated, *i.e.*, that glu-204 is the residue which releases the proton to the extracellular surface during the photocycle, as was indeed found later.

According to the scheme, therefore, near neutral pH either asp-85 or glu-204 can deprotonate with a low p$K_a$ but not both. When applied to the events in the photocycle, the lower of the two p$K_a$'s of glu-204 refers to its proton affinity after protonation of asp-85, while the higher of the two p$K_a$'s of asp-85 refers to its proton affinity after dissociation of glu-204. It was pointed out by Balashov et al. (1995, 1996) that interacting proton affinities between asp-85 and X′H could account for the proton release to the extracellular side upon protonation of asp-85 in the photocycle. The linkage therefore explains why the group that releases the proton in the photocycle has a high p$K_a$ in the unphotolyzed protein (predicted p$K_a$ = 9.2−9.4), in which asp-85 is unprotonated, but has a low p$K_a$ after photoisomerization (predicted p$K_a$ = 4.7) once asp-85 becomes protonated. Experimental values for these two p$K_a$ values (measured previously without knowledge of the identity of the release group, termed XH) are 9.3 (Kono et al., 1993) and 4.9−5.8 (Zimányi et al., 1992b; Cao et al., 1993a), respectively. Although the p$K_a$ of glu-204 after photoisomerization of the retinal might not be exactly as predicted from the properties of the unphotolyzed protein, the reason for proton release from this residue upon protonation of asp-85 is explained satisfactorily.

We note that because the interaction of asp-85 and glu-204 is mutual, in this scheme the p$K_a$ of asp-85 is also variable, and that makes the deprotonation of glu-204 a component of the reprotonation switch. When the pH is well above the p$K_a$ of glu-204, the proton release from this residue is energetically favored and unidirectional and will raise the p$K_a$ of asp-85. After photoisomerization of the retinal, the initially large p$K_a$ difference between the Schiff base and asp-85 approaches zero. Although its value is not known at this time, it seems reasonable that the p$K_a$ of asp-85 would have to become higher than 2.5, even while glu-204 is still protonated. We assume that the effect of glu-204 on the p$K_a$ of asp-85 under these conditions is similar, although probably not identical, to what the scheme yields in the unphotolyzed state. Upon the deprotonation of glu-204 during the photocycle, the scheme of linked proton affinities predicts therefore that the p$K_a$ of asp-85 will increase once again, also perhaps by as much as 5 pH units (inset of Figure 2A) over its already higher value. If this is so, *loss of proton from glu-204 will make asp-85 no longer suitable as a proton donor to the Schiff base in the protonation equilibrium, and the proton release to the extracellular surface thus constitutes the first phase of the reprotonation switch*. This mechanism provides a satisfactory molecular rationale for the earlier observation that the $M_2 \rightarrow M_1$ back-reaction was accompanied by proton uptake and for the model proposed on this basis in which these two M substates are separated by the proton release step (Zimányi et al., 1992b).

In the last step in the photocycle, asp-85 regains its low p$K_a$, and the scheme in Figure 2A requires that the proton that is lost at this time (Bousché et al., 1992; Souvignier & Gerwert, 1992) be transferred to glu-204 as the initial state of the protein recovers. This is apparently less effective when residue 204 is an aspartate because the O $\rightarrow$ BR reaction is slower in the E204D mutant (Brown et al., 1995a). Protonation of residue 204 cannot occur in E204Q at all, and we assume that in this mutant the proton is released from asp-85 directly to the extracellular surface, as in the wild type at pH values low enough to prevent dissociation of glu-204. The particularly slow rate of the proton release in E204Q (Brown et al., 1995a) is as expected, therefore, if glu-204 is indeed part of the proton conduction pathway to the extracellular surface.

*Means of Linking the p$K_a$'s of asp-85 and glu-204.* For assessing this proposed mechanism, it would be important to know the p$K_a$ of asp-85 at every stage of the photocycle. The unusually high C═O stretch frequency of the protonated asp-85 in the M intermediate (1761 $cm^{-1}$) suggested at first that its p$K_a$ is as low as 2.5 (Rothschild et al., 1981), *i.e.*, as in the unphotolyzed state. Reinterpretation of this frequency (Braiman et al., 1996) suggests, however, a decreased degree of hydration near asp-85 in the $M_2$ state, and its p$K_a$ at this time is estimated to be well over 10. This would be consistent with the linked proton affinity scheme that predicts that this p$K_a$ will be greatly elevated, and this could be accomplished by redistributing (or reorienting) the water dipoles in the hydrogen-bonded network in this region so as to favor solvation of the anionic glu-204 and, therefore, destabilization of the dissociated form of asp-85. The participation of bound water in this reaction is consistent with the observation that the largest deuterium isotope effect in the photocycle (about 6-fold) is for the last phase of the formation of the M intermediate (Liu, 1990; Cao et al., 1995), which depends on the rate of the $M_1 \rightarrow M_2$ reaction, associated with proton release in our proposed photocycle scheme (Zimányi et al., 1992b). This kind of a rearrangement of water dipoles between asp-85 and glu-204 might be the cause of the disruption of hydrogen bonding and other polar interactions in the environment around asp-85 suggested by Braiman et al. (1996). On the other hand, the C═O frequency of asp-85 is unchanged in the M intermediates of the R82Q (Brown et al., 1994) and E204Q (Brown et al., 1995) mutants, where glu-204 does not dissociate, and the p$K_a$ of asp-85 is not expected to rise. If arg-82 were to assume alternating positions in the photocycle, toward either asp-85 or glu-204 as suggested (Balashov et al., 1993; Scharnagl et al., 1995), its absence from the vicinity of asp-85 after deprotonation of the Schiff base would explain its lack of effect on the C═O frequency. Such a mechanism would provide another means for linking the p$K_a$'s of asp-85 and glu-204.

In a few mutants, such as F208R, D212N/D96N (Cao et al., 1995), and to a smaller extent, D85E (Heberle et al., 1993), the proton release from glu-204 is delayed, and presumably what would be the first phase of the reprotonation switch, the increase of the p$K_a$ of asp-85 (or glu-85), is also delayed. When dissociation of glu-204 cannot occur at all in the photocycle, either because the pH is below its p$K_a$ or because important residues in this region have been replaced as in the R82Q or A and E204Q mutants, the proposed mechanism predicts that the p$K_a$ of asp-85 will not rise. Under these conditions the reprotonation switch is not an irreversible reaction. Nevertheless, transport does take place. In these cases one would expect that asp-85 remains in equilibrium with the Schiff base until the end of the

photocycle, *i.e.*, the L intermediate remains in equilibrium with $M_1$, $M_2$, N, and O, until the final unidirectional reaction repopulates the initial unphotolyzed state of the protein. Indeed, in D96N at pH ≤ 5 (Zimányi et al., 1992b) and in E204Q,[2] where the kinetics were investigated in sufficient detail, this was so. The $M_1$ to $M_2$ reaction was not unidirectional, and the L intermediate persisted until the end of the photocycle.

*Mechanism of the Reprotonation Switch*. In light of the results reported here we propose that the reprotonation switch consists of three phases. The *first phase* occurs in the extracellular region, and its function is to make asp-85, once protonated, a poor proton donor to the unprotonated Schiff base. After the relative proton affinities of asp-85 and the Schiff base are changed in the photocycle so as to decrease their $\Delta pK_a$, and the proton equilibrates between the two groups, the redistribution of charges through a hydrogen-bonded network that includes arg-82 and bound water causes dissociation of a proton from glu-204 and its release to the extracellular surface. This raises the $pK_a$ of asp-85 much above the $pK_a$ of the Schiff base, and the protonation equilibrium is shifted strongly against proton transfer back to the Schiff base. The connection between the Schiff base and asp-85 is therefore broken by an "affinity" type mechanism.

The *second phase* of the reprotonation switch has the function to connect the Schiff base to asp-96. There is some evidence that this is accomplished by an "accessibility" type mechanism. At pH below the $pK_a$ for proton release the persistence of the L state during the entire lifetime of M (Zimányi et al., 1992b) indicates that when the $pK_a$ of asp-85 is not raised, presumably because glu-204 does not dissociate, the Schiff base cannot fully deprotonate. Nevertheless, even under these conditions the initial protonation equilibrium between the Schiff base and asp-85, achieved at about 10 $\mu$s, was found to be shifted at several hundred microseconds toward more complete deprotonation of the Schiff base. Unlike at higher pH, where proton release made this reaction virtually complete, the M to L ratio at pH < 6 was only 20:1. The origin of this L to M shift was not clear and was assigned to a component of the $M_1 \rightarrow M_2$ reaction that was not dependent on proton release (Zimányi et al., 1992b). This makes it distinct from the proposed first phase of the switch that is dependent on the dissociation of glu-204.

The $M_1 \rightarrow M_2$ reaction was detected as a 6−7 nm blue-shift of the absorption maximum during the lifetime of the M state in the D96N single mutant (Zimányi et al., 1992a) and in double mutants such as R82Q/D96N where a proton is not released (Cao et al., 1995). This wavelength shift is not observed in the wild type. We argued that the blue-shift of $M_2$, but not $M_1$, in the asp-96 mutants reflects a diminution in the hydrogen-bonding that normally causes a red-shift of the absorption maximum of the M state from the expected 380 nm to 411−412 nm. It follows that in the wild type the Schiff base becomes hydrogen-bonded to asp-96 through intervening water molecules in the cytoplasmic half-channel, at this time in the photocycle, which is consistent with a change of accessibility in $M_2$ from the extracellular to the cytoplasmic side. The blue-shift in D96N would originate from the different (and less suited) hydrogen-bonding geometry of the asparagine as residue 96. Thus, the spectral shift of the maximum of M suggests that asp-96 is connected to the Schiff base in $M_2$ but not in $M_1$.

The function of the *third phase* of the reprotonation switch is to change the relative $pK_a$'s of asp-96 and the Schiff base so as to induce proton transfer. The $pK_a$ of asp-96 is above 11 in the unphotolyzed state (Száraz et al., 1994) but is lowered to near 7 during the photocycle of a mutant protein, independent of the protonation state of the Schiff base (Cao et al., 1993b). Kinetic studies of the decay of $M_2$ in the D96N mutant (Brown & Lanyi, 1996) have indicated that the $pK_a$ of the Schiff base at this time is 8.3, *i.e.*, somewhat higher than that of asp-96. Analyses of the $M_2 \Leftrightarrow N$ reaction in the wild type photocycle (Zimányi et al., 1993) have been consistent with a $\Delta pK_a$ of this magnitude and sign between the Schiff base and asp-96. The estimated proton affinities of the Schiff base and asp-96 thus become at this time in the photocycle, but not before, appropriate for the reprotonation reaction.

*Roles of Protein Conformational Change*. What is then the function of protein conformation change in the proton transport? While the large-scale protein conformational shifts observed by diffraction (Koch et al., 1991; Nakasako et al., 1991; Subramaniam et al., 1993; Han et al., 1994; Kataoka et al., 1994; Kamikubo et al., 1996) do not seem to have a role in the first phase of the switch, their function may be to facilitate the second and third phases. The structural changes were suggested recently to consist of two sequential steps (Kamikubo et al., 1996). The first is the increased density observed at the cytoplasmic half of helices G and B. The strong influence of thr-46, a residue on helix B, on proton transfer from asp-96 to the Schiff base at this time in the photocycle has been amply demonstrated (Marti et al., 1991a; Brown et al., 1994; Yamazaki et al., 1995). This conformation change could therefore be the structural basis for the second phase of the switch. The density increase at helix B in the projection map is then replaced by a pair of negative and positive density changes near helix F that indicate the outward tilt of the cytoplasmic end of this helix. Changes of the vibrational frequency of the peptide C═O of tyr-185 (Ludlam et al., 1995) at this time had suggested that the well-known amide band changes observed in FTIR difference spectra of the $M_N$ (Sasaki et al., 1992) and N (Braiman et al., 1991; Ormos et al., 1992; Souvignier & Gerwert, 1992) intermediates might originate from this structural transition. The changes at helix G and the outward displacement of helix F indeed persist in the N state (Kamikubo et al., 1996). Opening of a cleft at the cytoplasmic end of helix F would allow increased hydration of the cytoplasmic half-channel and thereby lower the $pK_a$ of asp-96 and facilitate the proton transfer from this residue to the Schiff base, consistent with the effects of osmotic agents (Cao et al., 1991), lowered humidity (Váró & Lanyi, 1991b), and hydrostatic pressure (Váró & Lanyi, 1995). Introducing bulky groups to an engineered cysteine on helix F but not on the other helices caused more rapid reprotonation of the Schiff base but much more slow protonation of asp-96, and cross-linking at this location but not at the others had the opposite effect (Brown et al., 1995c). These results linked the proton transfers from asp-96 to the Schiff base and reprotonation of asp-96 from the cytoplasmic surface to the outward tilt of helix F and its recovery, respectively. The

[2] G. Váró and J. K. Lanyi, unpublished results.

final structural change in M and N, and thus the modulation of the p$K_a$ of asp-96 through changing hydration, should correspond therefore to the third phase of the reprotonation switch.

## ACKNOWLEDGMENT

We are grateful to Klaus Schulten for the coordinates of the structure in Figure 6 and for his permission to use them and to T. G. Ebrey, S. P. Balashov, and M. S. Braiman for valuable suggestions and pre-publication access to their manuscripts.

## REFERENCES

Balashov, S. P., Govindjee, R., & Ebrey, T. G. (1991) *Biophys. J. 60*, 475−490.

Balashov, S. P., Govindjee, R., Kono, M., Imasheva, E., Lukashev, E., Ebrey, T. G., Crouch, R. K., Menick, D. R., & Feng, Y. (1993) *Biochemistry 32*, 10331−10343.

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

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

Bitting, H. C., Jang, D.-J., & El-Sayed, M. A. (1990) *Photochem. Photobiol. 51*, 593−598.

Bousché, O., Sonar, S., Krebs, M. P., Khorana, H. G., & Rothschild, K. J. (1992) *Photochem. Photobiol. 56*, 1085−1095.

Braiman, M. S., Bousché, O., & Rothschild, K. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2388−2392.

Braiman, M. S., Dioumaev, A. K., & Lewis, J. R. (1996) *Biophys. J. 70*, 939−947.

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

Brown, L. S., Yamazaki, Y., Maeda, M., Sun, L., Needleman, R., & Lanyi, J. K. (1994) *J. Mol. Biol. 239*, 401−414.

Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., & Lanyi, J. K. (1995a) *J. Biol. Chem. 270*, 27122−27126.

Brown, L. S., Váró, G., Hatanaka, M., Sasaki, J., Kandori, H., Maeda, A., Sheves, M., Needleman, R., & Lanyi, J. K. (1995b) *Biochemistry 34*, 12903−12911.

Brown, L. S., Váró, G., Needleman, R., & Lanyi, J. K. (1995c) *Biophys. J. 69*, 2103−2111.

Brown, L. S., & Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1731−1734.

Cao, Y., Váró, G., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1991) *Biochemistry 30*, 10972−10979.

Cao, Y., Brown, L. S., Needleman, R., & Lanyi, J. K. (1993a) *Biochemistry 32*, 10239−10248.

Cao, Y., Váró, G., Klinger, A. L., Czajkowsky, D. M., Braiman, M. S., Needleman, R. & Lanyi, J. K. (1993b) *Biochemistry 32*, 1981−1990.

Cao, Y., Brown, L. S., Sasaki, J., Maeda, A., Needleman, R., & Lanyi, J. K. (1995) *Biophys. J. 68*, 1518−1530.

Chang, C.-H., Chen, J.-G., Govindjee, R., & Ebrey, T. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 396−400.

de Groot, H. J. M., Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1989) *Biochemistry 28*, 3346−3353.

de Groot, H. J. M., Smith, S. O., Courtin, J., Van den Berg, E., Winkel, C., Lugtenburg, J., Griffin, R. G., & Herzfeld, J. (1990) *Biochemistry 29*, 6873−6883.

Dér, A., Száraz, S., Tóth-Boconádi, R., Tokaji, Z., Keszthelyi, L., & Stoeckenius, W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4751−4755.

Dioumaev, A. K., & Braiman, M. S. (1995) *J. Am. Chem. Soc. 117*, 10572−10574.

Druckmann, S., Ottolenghi, M., & Korenstein, R. (1985) *Biophys. J. 47*, 115−118.

Druckmann, S., Ottolenghi, M., Rousso, I., Friedman, N., & Sheves, M. (1995) *Biochemistry 34*, 12066−12074.

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

Fodor, S. P., Ames, J. B., Gebhard, R., van der Berg, E. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. A. (1988) *Biochemistry 27*, 7097−7101.

Han, B.-G., Vonck, J., & Glaeser, R. M. (1994) *Biophys. J. 67*, 1179−1186.

Heberle, J., Oesterhelt, D., & Dencher, N. A. (1993) *EMBO J. 12*, 3721−3727.

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

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

Jonas, R., & Ebrey, T. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 149−153.

Kalisky, O., Ottolenghi, M., Honig, B., & Korenstein, R. (1981) *Biochemistry 20*, 649−655.

Kamikubo, H., Kataoka, M., Váró, G., Oka, T., Tokunaga, F., Needleman, R., & Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1386−1390.

Kandori, H., Yamazaki, Y., Sasaki, J., Needleman, R., Lanyi, J. K., & Maeda, A. (1995) *J. Am. Chem. Soc. 117*, 2118−2119.

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

Koch, M. H. J., Dencher, N. A., Oesterhelt, D., Plöhn, H.-J., Rapp, G., & Büldt, G. (1991) *EMBO J. 10*, 521−526.

Kono, M., Misra, S., & Ebrey, T. G. (1993) *FEBS Lett. 331*, 31−34.

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

Lanyi, J. K. (1995) *Nature 375*, 461−463.

Liu, S. Y. (1990) *Biophys. J. 57*, 943−950.

Ludlam, C. F. C., Sonar, S., Lee, C.-P., Coleman, M., Herzfeld, J., RajBhandary, U. L., & Rothschild, K. J. (1995) *Biochemistry 34*, 2−6.

Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462−467.

Maeda, A., Sasaki, J., Yamazaki, Y., Needleman, R., & Lanyi, J. K. (1994) *Biochemistry 33*, 1713−1717.

Marti, T., Otto, H., Mogi, T., Rösselet, S. J., Heyn, M. P., & Khorana, H. G. (1991a) *J. Biol. Chem. 266*, 6919−6927.

Marti, T., Rösselet, S. J., Otto, H., Heyn, M. P., & Khorana, H. G. (1991b) *J. Biol. Chem. 266*, 18674−18683.

Metz, G., Siebert, F., & Engelhard, M. (1992) *FEBS Lett. 303*, 237−241.

Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, Y. W., Taylor, M., & Stoeckenius, W. (1979) *Biochemistry. 18*, 4100−4107.

Nagle, J. F., & Mille, M. (1981) *J. Chem. Phys. 74*, 1367−1372.

Nakasako, M., Kataoka, M., Amemiya, Y., & Tokunaga, F. (1991) *FEBS Lett. 292*, 73−75.

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

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

Ohno, K., Takeuchi, Y., & Yoshida, M. (1977) *Biochim. Biophys. Acta 462*, 575−582.

Ormos, P., Chu, K., & Mourant, J. (1992) *Biochemistry 31*, 6933−6937.

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

Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.

Rothschild, K. J., Zagaeski, M., & Cantore, W. A. (1981) *Biochem. Biophys. Res. Commun. 103*, 483−489.

Sasaki, J., Shichida, Y., Lanyi, J. K., & Maeda, A. (1992) *J. Biol. Chem. 267*, 20782−20786.

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

Schulten, K., Schulten, Z., & Tavan, P. (1984) in *Information and Energy Transduction in Biological Membranes* (Bolis, A., Helmreich, H., & Passow, Eds.) pp 113−131, H. Alan R. Liss, Inc., New York.

Souvignier, G., & Gerwert, K. (1992) *Biophys. J. 63*, 1393−1405.

Subramaniam, S., Marti, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1013−1017.

Subramaniam, S., Greenhalgh, D. A., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 25730−25733.

Subramaniam, S., Gerstein, M., Oesterhelt, D., & Henderson, R. (1993) *EMBO J. 12*, 1−8.
Száraz, S., Oesterhelt, D., & Ormos, P. (1994) *Biophys. J. 67*, 1706−1712.
Thorgeirsson, T. E., Milder, S. J., Miercke, L. J. W., Betlach, M. C., Shand, R. F., Stroud, R. M., & Kliger, D. S. (1991) *Biochemistry 30*, 9133−9142.
Tittor, J., Schweiger, U., Oesterhelt, D., & Bamberg, E. (1994) *Biophys. J. 67*, 1682−1690.
Turner, G. J., Miercke, L. J. W., Thorgeirsson, T. E., Kliger, D. S., Betlach, M. C., & Stroud, R. M. (1993) *Biochemistry 32*, 1332−1337.
Váró, G., & Lanyi, J. K. (1989) *Biophys. J. 56*, 1143−1151.
Váró, G., & Lanyi, J. K. (1990) *Biochemistry 29*, 2241−2250.
Váró, G., & Lanyi, J. K. (1991a) *Biochemistry 30*, 5016−5022.
Váró, G., & Lanyi, J. K. (1991b) *Biophys. J. 59*, 313−322.
Váró, G., & Lanyi, J. K. (1995) *Biochemistry 34*, 12161−12169.
Váró, G., Zimányi, L., Fan, X., Sun, L., Needleman, R., & Lanyi, J. K. (1995) *Biophys. J. 68*, 2062−2072.
Yamazaki, Y., Hatanaka, M., Kandori, H., Sasaki, J., Karstens, W. F. J., Raap, J., Lugtenburg, J., Bizounok, M., Herzfeld, J., Needleman, R., Lanyi, J. K., & Maeda, A. (1995) *Biochemistry 34*, 7088−7093.
Zimányi, L., Cao, Y., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992a) *Photochem. Photobiol. 56*, 1049−1055.
Zimányi, L., Váró, G., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992b) *Biochemistry 31*, 8535−8543.
Zimányi, L., Cao, Y., Needleman, R., Ottolenghi, M., & Lanyi, J. K. (1993) *Biochemistry 32*, 7669−7678.

BI952883Q