Arginine-82 Regulates the pK_a of the Group Responsible for the Light-Driven Proton Release in Bacteriorhodopsin

Rajni Govindjee,* Saurav Misra,* Sergei P. Balashov,* Thomas G. Ebrey,*# Rosalie K. Crouch,§ and Donald R. Menick§

*Center for Biophysics and Computational Biology and #Department of Cell and Structural Biology, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, and §Medical University of South Carolina, Charleston, South Carolina 29425 USA

ABSTRACT In wild-type bacteriorhodopsin light-induced proton release occurs before uptake at neutral pH. In contrast, in mutants in which R82 is replaced by a neutral residue (as in R82A and R82Q), only a small fraction of the protons is released before proton uptake at neutral pH; the major fraction is released after uptake. In R82Q the relative amounts of the two types of proton release, "early" (preceding proton uptake) and "late" (following proton uptake), are pH dependent. The main conclusions are that 1) R82 is not the normal light-driven proton release group; early proton release can be observed in the R82Q mutant at higher pH values, suggesting that the proton release group has not been eliminated. 2) R82 affects the pK_a of the proton release group both in the unphotolyzed state of the pigment and during the photocycle. In the wild type (in 150 mM salt) the pK_a of this group decreases from ~9.5 in the unphotolyzed pigment to ~5.8 in the M intermediate, leading to early proton release at neutral pH. In the R82 mutants the respective values of pK_a of the proton release group in the unphotolyzed pigment and in M are ~8 and 7.5 in R82Q (in 1 M salt) and ~8 and 6.5 in R82K (in 150 mM KCl). Thus in R82Q the pK_a of the proton release group does not decrease enough in the photocycle to allow early proton release from this group at neutral pH. 3) Early proton release in R82Q can be detected as a photocurrent signal that is kinetically distinct from those photocurrents that are due to proton movements from the Schiff base to D85 during M formation and from D96 to the Schiff base during the M → N transition. 4) In R82Q, at neutral pH, proton uptake from the medium occurs during the formation of O. The proton is released during the O → bacteriorhodopsin transition, probably from D85 because the normal proton release group cannot deprotonate at this pH. 5) The time constant of early proton release is increased from 85 μs in the wild type to 1 ms in R82Q (in 150 mM salt). This can be directly attributed to the increase in the pK_a of the proton release group and also explains the uncoupling of proton release from M formation. 6) In the E204Q mutant only late proton release is observed at both neutral and alkaline pH, consistent with the idea that E204 is the proton release group. The proton release is concurrent with the O → bacteriorhodopsin transition, as in R82Q at neutral pH.

Introduction

Bacteriorhodopsin (bR), the chromoprotein present in the purple membrane of *Halobacterium salinarium*, contains a retinal molecule attached by means of a protonated Schiff base linkage to K216 of the protein. On excitation with light, bR undergoes a photocycle consisting of a sequence of spectroscopically distinct intermediates, K, L, M, N and O, before returning to the original state. During the photocycle, there is a net vectorial transport of a proton from the cytoplasmic to the extracellular side of the membrane (Lozier et al., 1975). The light-induced chromophore isomerization from an all-*trans* to a 13-*cis* configuration leads to a decrease in the pK_a of the Schiff base (Govindjee et al., 1994). As a result the proton from the protonated Schiff base is transferred to the internal proton acceptor D85 during the L → M transition. The Schiff base is subsequently reprotonated by another aspartate residue, D96, during the M → N transition (see reviews by Oesterhelt et al., 1992; Rothschild, 1992; Ebrey, 1993; Khorana, 1993; Lanyi, 1993). D85 stays protonated until the end of the photocycle (Siebert et al., 1982; Braiman et al., 1988).

In wild-type (WT) bR at neutral pH the intramolecular proton transfer from the protonated Schiff base to D85 during the L → M transition is coupled to proton release (hereafter referred to as early or fast proton release, $\tau = 85$ μs) on the extracellular surface of the membrane from a group called *X* (Siebert et al., 1982; Zimányi et al., 1992; Ebrey, 1993), followed by a slower proton uptake ($\tau \approx 5$ ms) on the cytoplasmic surface during the N → O transition. The pK_a of *X* in the M state is 5.8 in 150 mM KCl (Váro and Lanyi, 1990; Zimányi et al., 1992) and 4.6–4.9 in 2 M NaCl (Cao et al., 1993). At low pH ($pH < pK_a$), *X* cannot deprotonate, and proton release is delayed until the O → bR transition (Lanyi, 1993; Cao et al., 1993) in which D85 deprotonates, possibly directly into the extracellular medium (Gerwert et al., 1990; Rothschild, 1992; Souvignier and Gerwert, 1992; also see Fig. 7 below). Thus, at low pH, proton release is delayed until after proton uptake (Zimányi et al., 1992); we will refer to this as late proton release.

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Address reprint requests to Dr. Thomas G. Ebrey, Department of Cell and Structural Biology, University of Illinois, 506 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801. Tel.: 217-333-2015; Fax: 217-244-6615; E-mail:tebrey@ux1.cso.uiuc.edu.

Abbreviations: bR, bacteriorhodopsin; WT, wild type. Bacteriorhodopsin mutants are designated R82Q, R82K, etc., where the first letter and number represent the wild-type residue and the second letter represents the substituted residue.

This paper is dedicated to Pill-Soon Song on the occasion of his 60th birthday.

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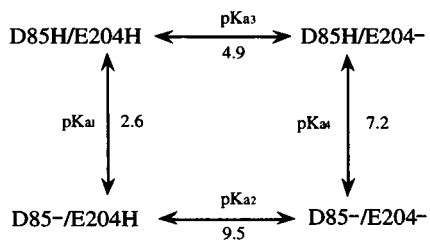
The proton release process is strongly affected by substitution of some of the residues, such as R82 and Y57, present in the extracellular domain of the proton channel (Henderson et al., 1990). In the R82Q, R82A, and Y57F mutants at neutral pH, proton release is drastically delayed and occurs after proton uptake (Otto et al., 1990; Balashov et al., 1992; 1993; Cao et al., 1993; Govindjee et al., 1995); if R82 is replaced with a positively charged lysine residue, the normal order of proton release before uptake is restored at neutral pH (Balashov et al., 1995). This suggests that these residues are either directly or indirectly involved in the early proton release process. A second effect of the replacement of R82 or Y57 with a neutral residue is to increase the pK_a of D85 from 2.6 in the WT to 7.2 in R82A and R82Q (Stern and Khorana, 1989; Otto et al., 1990; Subramaniam et al., 1990; Thorgeirsson et al., 1991; Balashov et al., 1993, 1995; Cao et al., 1993) and to 3.8 and 4.7 in Y57N and Y57F, respectively (Govindjee et al., 1995).

The complete titration curves of D85 in the WT and in R82K suggested that D85 interacts with a residue, X', whose pK_a is $\sim 9.5 \pm 0.3$ in the WT and 8.0 ± 0.2 in R82K (Balashov et al., 1995; 1996). X' was identified by Richter et al. (1996) to be E204. From titrations performed on the unphotolyzed pigments, the pK_a of this residue was inferred to decrease to ~4.9 in the WT (~5.7 in R82K) on protonation of D85, as depicted in the following scheme:



Such an interrelationship between D85 and X' suggested that in an analogous situation during the photocycle, on formation of the M intermediate (in which D85 also becomes protonated), the pK_a of X' should drop, causing it to release a proton. That is, X' possesses that quality required of the proton release group X of having its pK_a greatly lowered by the protonation of D85. Balashov et al. (1993, 1995, 1996) suggested that X' (identified through its effect on the titration of D85 in the unphotolyzed state) and X (defined as the terminal group that releases the proton detected in the bulk in the photocycle during the M intermediate) represent complementary aspects of the same group. They also suggested the possibility that this group might be R82 or alternatively that R82 greatly affects the pK_a of this group. Based on theoretical calculations Scharnagl et al. (1994, 1995) suggested that X could be E204. Using Fourier-transform IR spectroscopy, Brown et al. (1995a) obtained experimental evidence for the deprotonation of E204 on M formation, indicating that X is actually E204.

The protonation state or the pK_a of E204 or both can thus be investigated by the use of complementary techniques for the unphotolyzed state of the pigment and during the photocycle. Titration of D85 in the unphotolyzed state yields two values of pK_a for E204, corresponding (as stated above) to the forms of the pigment in which D85 is protonated (the blue membrane) or deprotonated (the purple membrane) (Balashov et al. 1993, 1995, 1996). The lower pK_a, corresponding to the pK_a of E204 in the unphotolyzed state when D85 is protonated, allows a rough estimate of the pK_a of E204 in the M intermediate. Note that the states of the pigment are not identical; the blue membrane contains all-*trans* retinal and a protonated Schiff base, whereas the M intermediate contains 13-*cis* retinal and a deprotonated Schiff base; in both, D85 is protonated. Nevertheless, we noted that, in the WT, these two values of pK_a were separated by only 1 unit: 4.8 in the blue membrane (Balashov et al., 1996) and 5.8 during M (Zimányi et al., 1992). The actual pK_a of E204 in M can be measured directly by titrating the relative amounts of early or late proton release or both for a pigment (Zimányi et al, 1992), because thermodynamically E204 cannot release a proton during M (early proton release) when the pH is below its pK_a. In addition, the kinetics of M formation are influenced by the protonation state (in the unphotolyzed pigment) of a residue with a pK_a of ~9.2 in 150 mM KCl (Balashov et al, 1991). This residue has been suggested to be the proton release group in the unphotolyzed state of the pigment (Kono et al, 1993); thus, a pH-dependent change of the kinetics of M formation can be used to determine the pK_a of E204 in the unphotolyzed pigment. This calculation should yield a value similar to the high pK_a of E204 calculated from titrations of D85 in the unphotolyzed state (as discussed above); in practice, this has been found to be the case for the WT (Kono et al. 1993; Balashov et al, 1996; see Discussion).

In this study we have investigated the role of R82 in proton release in bR, in particular the effect of R82 replacement on the pH dependence of proton release. In the WT pigment the order of proton release and uptake is reversed if the pH of the medium is lower than the pK_a of the proton release group in the M intermediate (Zimányi et al., 1992). Thus, one explanation for the reversed order of proton release and uptake in R82 mutants is that the replacement of R82 with a neutral residue (as in R82Q and R82A) increases the pK_a of the proton release group (during M) so that early proton release becomes thermodynamically unfeasible. To investigate the possibility that R82 affects the pK_a of the proton release group, we studied the pH dependence of the kinetics of proton release and uptake in R82Q and R82K mutants, using pH-sensitive dyes and photocurrent measurements. We found that the pK_a of the proton release group (in the M state) is elevated by 2.5 units in R82Q compared with that in the WT, which explains the reversed order of proton uptake and release at neutral pH. A 1-ms (3 ms in 25% glycerol) photocurrent component attributed to the proton release process is described for the R82Q mutant. The pK_a of E204 (in M) is also increased in R82K but to a

lesser extent than in R82Q, suggesting that the influence of R82 on the E204 is at least partially due to electrostatic interactions. Thus R82 affects the pK_a not only of D85 but also of the proton release group E204.

MATERIALS AND METHODS

Site-directed mutagenesis of bR and transformation of R82Q and R82K mutants into *H. salinarium* strain IV-8 (which contains an ISH1 insert within the *bop* gene) and the preparation of the purple membrane were as described in Balashov et al. (1993, 1995). Flash-induced transient absorption changes were measured with a home-built kinetic spectrophotometer (Govindjee et al., 1990). Actinic flashes (532-nm, 7-ns pulses, 5–10 mJ/pulse) were provided from a Quanta Ray DCR-11 Nd:YAG laser (Spectra-Physics, Mountain View, CA); flash intensity was adjusted to achieve 10–15% photocycling.

pH indicator dye measurements

Depending on the pH of the measurement, we monitored proton release and uptake by measuring the transient absorption changes of one of three different pH-sensitive dyes, pyranine (at 460 nm), *p*-nitrophenol (at 400 nm), or umbelliferone (at 380 nm). The pK values of the dyes in 1 M and 150 mM NaCl, respectively, are 6.7 and 7.2 for pyranine, 6.6 and 6.8 for *p*-nitrophenol, and 7.5 and 7.8 for umbelliferone. We obtained the absorption change of the dye by subtracting the kinetic traces in the presence and in the absence of the dye as described previously (Govindjee et al., 1980).

An alternative method to obtain the dye absorption kinetics by subtracting two kinetic traces, both with the dye present but one with and the other without buffer, was found to be unsuitable, because the dye absorbance changes overlap that of the M photointermediate and the M decay kinetics are affected by the presence of buffers (even if the salt concentration is kept constant). The time constant of the fast component of M decay, representing the reprotonation of the Schiff base by D96 in the M → N transition, is unaffected by the addition of buffers, whereas the time constant of the slow component, which has been attributed to a back reaction from N to M (Chernovskii et al., 1989; Otto et al., 1989; Váró and Lanyi, 1990), decreases on addition of buffers, particularly at pH > 8 (data not shown). The sensitivity of M decay to buffers means that one cannot reliably obtain proton kinetics by this method.

Inasmuch as the pK_a of D85 (the purple-to-blue transition) in R82Q is ~6.5 in 1 M salt (see Results), most of the measurements were carried out in 1 M NaCl/25% glycerol suspensions; under these conditions a large fraction of the sample is in the purple state, providing the largest possible dye signal. Addition of glycerol (which slows down photocycle and dye kinetics) was necessitated by the tendency of the samples to aggregate in 1 M salt solution. Curve fitting was performed with the built-in routines of the Kaleidagraph software package (Synergy Software, Reading, PA) and our own programs. Dye traces were fitted as sums of two negative exponential components (representing the early and the late proton release phases) and one positive component (representing the proton uptake phase), with the added constraint that the amplitudes of the positive and negative phases must sum to zero (theoretical curves generated using these premises and utilizing typical values for the time constants of these phases are shown in Fig. 1 *B* and *C*). Kinetic absorbance traces were fitted as sums of exponentials.

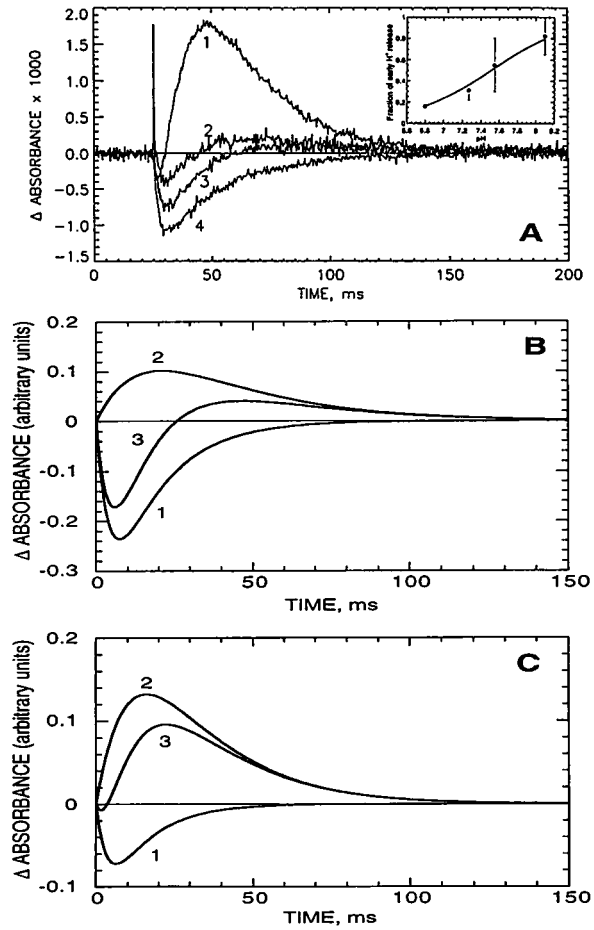


FIGURE 1 (*A*) Flash-induced proton changes as a function of pH in R82Q as measured with pH-sensitive dyes. Trace 1, Δ*A* pyranine at pH 7 showing a small negative signal that is due to early proton release (τ = 3.5 ± 0.4 ms, ~16% of the total proton release) followed by a large positive signal that is due to proton uptake (τ = 13 ± 1.8 ms), which precedes a late proton release (τ = 23 ± 2 ms, ~84% of the total proton release). Traces 2–4, ΔA umbelliferone, showing increased amplitude of the early proton release phase: trace 2, pH 7.3 (early proton release/total ~34%); trace 3, pH 7.6 (early proton release/total ~55%); trace 4, pH 8.1 (early proton release/total ~81%). Dye traces have been adjusted to reflect the same amount of M. *Inset*, the pH dependence of the fraction of early proton release, pK_a ~7.5. $\lambda_{actinic}$ 532 nm, $\lambda_{monitoring}$ 460 nm (for pyranine) and 380 nm (for umbelliferone), 1 M NaCl/25% glycerol, 20°C. (*B*), (*C*) Examples of the deconvolution of the proton kinetic traces to obtain the relative contributions of early and late proton release as described in Methods. (*B*) Curve 1, 50% of early proton release (τ = 3.5 ms) followed by proton uptake (τ = 13 ms); curve 2, 50% late proton release (τ = 20 ms) preceded by proton uptake (τ = 13 ms); curve 3 (sum of curves 1 and 2), expected proton kinetics, which reproduces trace 3 of (*A*). (*C*) Curve 1, 16% early proton release followed by uptake; curve 2, 84% late proton release preceded by uptake; curve 3 (sum of curves 1 and 2), expected proton kinetics, which reproduces trace 1 of (*A*).

Photocurrent measurements

We measured flash-induced absorption changes at 410 nm and photocurrents in gels containing oriented R82Q membranes (11 μM), using a homemade apparatus described by Liu and Ebrey (1988). The actinic flash was provided by the Nd:YAG laser mentioned above. Gel pieces were prepared by the method of Dér et al. (1985), modified as described in Liu (1990). The samples were soaked with a much lower salt concentration than was used for the pH dye experiments, because the photocurrent signal-to-noise ratio decreases in solutions of high ionic strength. Individual gel pieces were each soaked in 100 mM KCl/50 mM Tris buffer for two days. We preadjusted the pH of each solution by mixing appropriate amounts of 100 mM KCl/50 mM Tris acid and base solutions to reach a total volume of ~12 ml (2 ml for samples in D_2O). Sample pH values were remeasured just before the photocurrent and absorbance experiments. Photocurrents were amplified with 100-kΩ and 1-MΩ converters described by

Liu and Ebrey (1988). Curve fitting of the photocurrent traces was performed by use of the built-in routines of the Kaleidagraph software package. All the traces were fitted as sums of decaying exponentials.

RESULTS

pH dependence of proton release and uptake in R82Q

In WT bR at neutral pH, light causes an early proton release followed by proton uptake. In contrast, we find that, in R82Q at neutral pH, light-induced proton uptake occurs first, followed by a late (tens of milliseconds) proton release (Fig. 1 *A*, *trace 1*). This is consistent with earlier reports on R82 mutants (Otto et al., 1990; Balashov et al., 1993; Cao et al., 1993). A careful examination of the dye absorbance kinetics reveals that a small negative component ($\tau = 3.5 \pm 0.4$ ms), which can be ascribed to early proton release, is always present at neutral pH and constitutes almost 16% of the total proton signal (Fig. 1 *A*, *trace 1*). As the pH is increased, the fraction of the early proton release component increases while that of the late proton release ($\tau = 24 \pm 0.2$ ms) decreases (Fig. 1 *A*, *traces 2–4*).

Because the pK_a of pyranine in 1 M NaCl is 6.7, pyranine becomes unsuitable for use as an indicator at high pH. Therefore we measured the proton kinetics by using umbelliferone ($pK_a = 7.5$) as the indicator at pH > 7. Fig. 1 *A* (*traces 2–4*) shows flash-induced absorbance changes of umbelliferone from pH 7.3 to 8.1. At pH > 7 the dye signal that is due to early proton release increases from ~16% at pH 7 to ~80% at pH 8 (Fig. 1 *A*); this suggests that the pK_a of the proton release group, E204 (whose deprotonation is believed to be the source of the early proton release), is $\approx 7.5 \pm 0.3$ in 1 M NaCl (Fig. 1 *A*, *inset*). This pK_a is expected to be salt dependent because of the surface potential of the membrane; for example, in 150 mM KCl at pH 7.9 most of the protons are released after uptake, suggesting that the apparent pK_a of E204 (in M) is greater than 7.9 at this salt concentration (data not shown). At pH 7.9 in 150 mM KCl, the dye signals when either pyranine or umbelliferone is used are indentical (data not shown).

Interestingly, in the E204Q mutant (in which the proton release group has ostensibly been removed) flash-induced absorbance changes of pyranine at pH 7 (in 1 M NaCl/25% glycerol) show only late proton release, $\tau = 610$ ms, preceded by proton uptake, $\tau = 80$ ms (Fig. 2, *trace 1*); this result is in agreement with that of Brown et al. (1995a). At pH 8, absorbance changes of umbelliferone are very small but also show only late proton release (Fig. 2, *trace 3*), reinforcing the notion that the absence of early proton release at neutral pH is not due to the proton release group's having an elevated pK_a but is instead due to the absence of the proton release group. That is, these results offer further support that the group responsible for early proton release is E204.

Correlation of proton uptake and release in R82Q with the photocycle intermediates

To understand the nature of the proton transport pathway in the R82 mutants we have analyzed and compared the rates of proton uptake and release with those of the photocycle intermediates. Inasmuch as the rates of proton uptake and release in R82Q are quite slow at neutral pH, we can ignore the early photocycle intermediates, J, K, L, and M formation. Thus, we have focused on the events in the so-called second half of the photocycle, including M decay and the N and O intermediates. We have determined the kinetics of the M and O intermediates by measuring the absorption changes at 400 and 680 nm, respectively.

Correlations with M formation and decay

The pK_a of the purple-to-blue transition in R82Q is ~6.5 in 1 M salt (data not shown). As in the WT, the yield of M in R82Q is pH dependent and correlates with the amount of the purple form of the pigment. As shown in the *inset* of Fig. 3, the pK_a of the purple-to-blue transition determined from the amplitude of the M intermediate is also ~6.5 in 1 M NaCl/25% glycerol, compared with 7.2 in 150 mM salt (Fig. 5 *C*; Brown et al., 1993; Richter et al., 1996).

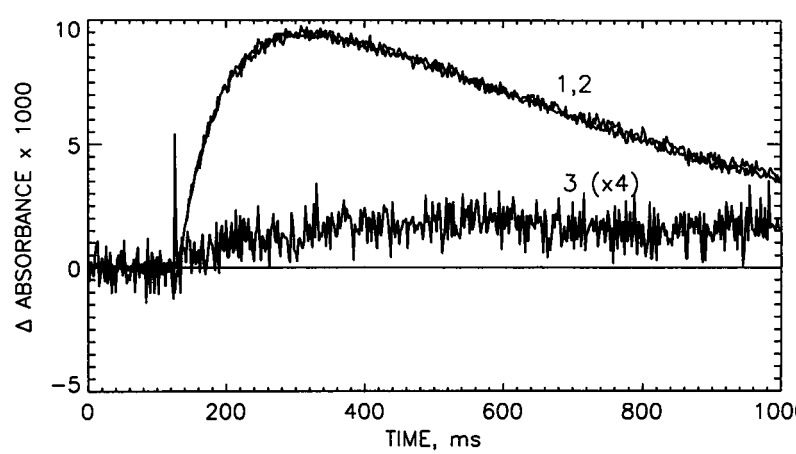


FIGURE 2 Flash-induced absorbance changes in E204Q. Trace 1: ΔA pyranine at pH 7, showing proton uptake ($\tau \approx 80$ ms) followed by late proton release ($\tau \approx 610$ ms). Trace 2: photointermediate O formation and decay measured at 680 nm at pH 7; the trace was obtained in the same manner as the equivalent in R82Q (see the Fig. 4 legend; also see Results). Trace 3: ΔA umbelliferone at pH 8. 1 M NaCl/25% glycerol.

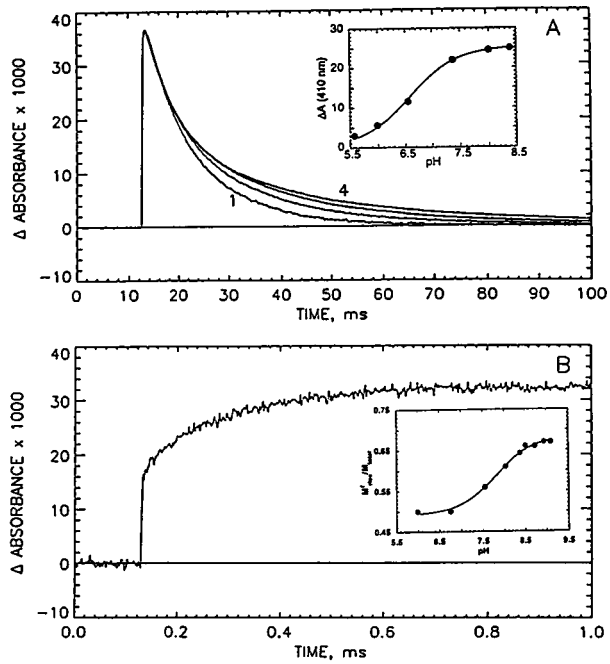


FIGURE 3 Flash-induced absorption changes in R82Q at 40 nm owing to the M photointermediate. (*A*) M decay as a function of pH, showing biphasic kinetics. Traces 1, 2, 3, and 4 at pH 7, 7.3, 7.8, and 8.1, respectively (all the traces have been normalized at Amax). τ_1 = 7 ± 0.4 ms is pH independent, amp$_1$ varies from 40% at pH 7 to 60% at pH 8.1; τ_2 varies from 13 ± 0.4 ms at pH 7 to 32 ± 0.2 ms at pH 8.1 and is pH dependent, amp$_2$ decreases from 60% at pH 7 to 40% at pH 8.1. The *inset* shows that the total M amplitude is pH dependent, pK$_a$ ~ 6.5. (*B*) The M rise is also biphasic, τ_1 ~ 1 μs (unresolved) and τ_2 = 160 ± 3 μs at pH 7. The *inset* shows that the fraction of M^f_{rise} is pH dependent, pK$_a$ ~ 8. 1 M NaCl/25% glycerol, 20°C.

The M decay kinetics in R82Q at neutral pH, in 1 M NaCl/25% glycerol, are biphasic (τ_1 = 7 ± 0.4 ms and τ_2 = 13 ± 0.4 ms). As shown in Fig. 3 *A*, the time constant of the fast-decaying component remains almost unchanged with pH, although its amplitude increases (from ~40% at pH 7 to ~60% at pH 8), whereas the time constant of the slow component becomes progressively slower ($\tau_2 \approx$ 32 ± 0.2 ms at pH 8) and its amplitude decreases from 60% to 40% between pH 7 and pH 8. A similar pH dependence of the amplitude of M decay components has been reported for the WT, with pK$_a$ ~8.0 in 100 mM NaCl (Li et al., 1984; Cao et al., 1993).

Fig. 3 *B* shows that M formation in R82Q in 1 M NaCl/25% glycerol, pH 7, is biphasic, $\tau_1 \approx$ 1 μs (unresolved in the time scale used) and $\tau_2 \approx$ 160 μs. At high pH, the relative amplitude of the fast phase of the M rise (M^f_{rise}) increases, whereas that of the slow phase decreases with a pK$_a \approx$ 8.1 (Fig. 3 *B*, *inset*). A change in the kinetics of M formation was previously ascribed to the deprotonation of a residue, possibly the proton release group, at high pH (Balashov et al., 1991; Kono et al., 1993); thus, this pK$_a$ value presumably represents the pK$_a$ of E204 in the unphotolyzed state (see Discussion).

The time constant of early proton release at pH 8.0 (τ = 3.5 ± 0.4 ms) is much longer than the time constants of Schiff base deprotonation and M rise (1 and 160 μs; Fig. 3 *B*) and somewhat faster than the fast phase of M decay (τ = 7 ± 0.4 ms). This implies that early proton release in R82Q follows but is not strictly coupled to Schiff base deprotonation.

The kinetics of proton uptake coincide with the slow phase of M decay in the pH range 7–8. At pH 7 the time constant of the slow component of M decay is the same as the time constant of proton uptake (Fig. 3 *A*, M decay τ_2 = 13 ± 0.4 ms; Fig. 1 *A*, proton uptake τ = 13 ± 1.8 ms). Proton uptake becomes slower at higher pH (τ = 31 ± 2 ms at pH 8.0) but still coincides with the slow phase of M decay (τ = 32 ms at pH 8.1). The slow phase of M decay essentially represents the {M ⇔ N} → O transition, as the M and N intermediates are thought to be in equilibrium late in the photocycle (Ames and Mathies, 1990; Druckmann et al., 1993); hence under these circumstances proton uptake would be expected to also correlate with the rise of the O intermediate.

Correlations with O formation and decay

Compared with that in the WT, in R82Q the O intermediate does not accumulate in large quantities (see also Cao et al., 1993); however, a small amount of O intermediate is observed at pH 7 in 1 M NaCl. The kinetics of the O intermediate were measured at 680 nm; at this wavelength only the absorbance changes that are due to O (positive component) and bR (negative component) are observed (Fig. 4 *A*, *trace 2*). Possible kinetic complications from the long-wavelength K intermediate of any 13-*cis* pigment present can be ignored because the decay of that intermediate is much faster than the O kinetics, as shown in Fig. 4 *A*, *trace 2* (the initial positive spike is due to the *batho* product of the 13-*cis* pigment). We deduced the absorbance change that is due to the O intermediate alone by subtracting an appropriately scaled signal resulting from the bleaching of bR (obtained by normalizing Δ*A* 590 nm, which is due mostly to transient bleaching and recovery of bR, with the initial decrease in absorbance at 680 nm; Fig. 4 *A*, *trace 1*) from the flash-induced absorption change at 680 nm (Fig. 4 *A*, *trace 2*). The resultant absorbance change representing the O intermediate is shown in Fig. 4 *A* (*trace 3*). The validity of the deduced absorbance changes of O is enhanced by the correlation between the kinetics of O formation (time constant of 14 ms) and the slow phase of M decay (13 ± 0.4 ms), as would be expected if the M and N intermediates equilibrate before decaying into the O intermediate.

A comparison of the kinetics of O formation and decay with those of the proton uptake and release, as observed with pyranine, is shown in Fig. 4 *B*. Under these conditions (1 M NaCl/25% glycerol, pH 7) the rates of proton uptake and release coincide with the rates of formation and decay of the O intermediate. This suggests that a proton is taken up

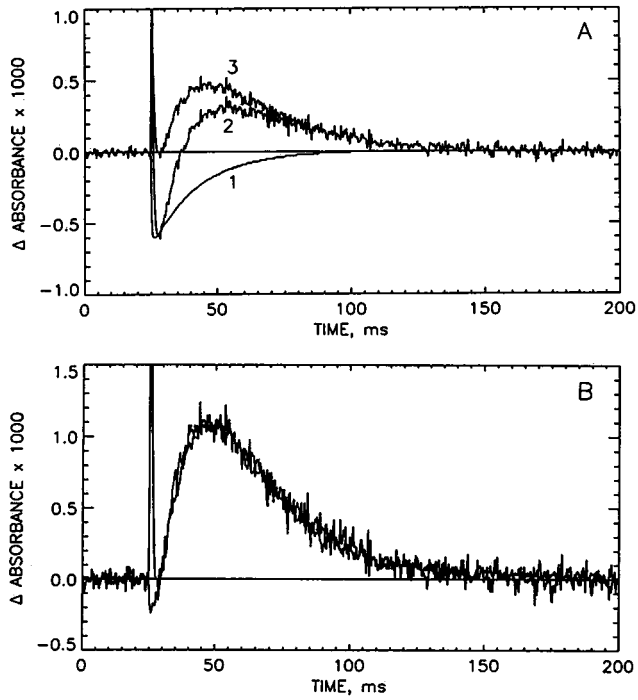


FIGURE 4 (*A*) Flash-induced absorption changes in R82Q. Trace 1, ΔA 590 nm (normalized to ΔA 680 nm at A_{min}); trace 2, ΔA 680 nm, showing the absorbance decrease of bR and the absorbance increase that is due to O intermediate; trace 3 (*trace 2 − trace 1*), ΔA 680 nm that is due to the O intermediate alone. (*B*) ΔA pyranine and ΔA 680 nm (*trace 3* of (*A*)) normalized at A_{max}, showing the coincidence of proton uptake ($\tau \approx 13$ ms) and release ($\tau \approx 24$ ms) with the formation and the decay, respectively, of the intermediate. 1 M NaCl/25% glycerol, pH 7, 20°C.

from the outside medium during the $\{M \Leftrightarrow N\} \rightarrow O$ transition. Proton release, which in the WT usually correlates with the $L \rightarrow M$ transition and the deprotonation of the proton release group, is delayed in R82Q and correlates with the $O \rightarrow bR$ transition; it presumably involves deprotonation of D85 (Gerwert et al., 1990). A similar coincidence between proton uptake and release and O formation and decay is also observed in the E204Q mutant (Fig. 2, *traces 1* and *2*).

Photocurrents in R82Q corresponding to early proton release

Fig. 5 *A* depicts the photocurrents seen in gel samples of R82Q at different pH values (in 100 mM KCl/50 mM Tris) in the same time domain as the early proton release and proton uptake seen in the dye measurements. These photocurrent signals are readily distinguished from the very fast photocurrents seen at shorter time scales (Fig. 5 *B*), which correlate strongly with the microsecond increase in absorbance at 410 nm, i.e., M formation (Balashov et al., 1993). As such, the faster (1 and 20 μs in 100 mM KCl/50 mM Tris) photocurrents most probably reflect the movement of the Schiff base proton to D85 (Keszthelyi and Ormos, 1980; Liu, 1990). On the millisecond time scale two positive photocurrent phases, decaying with time constants of 1 and 6 ms, respectively, are evident (Fig. 5 *A*). The 6-ms photocurrent correlates well with the faster, 7-ms component of M decay (Fig. 3 *A*) and most likely reflects the internal transfer of a proton from D96 to the Schiff base during N formation (see, for example, the review by Lanyi, 1993). The 1-ms photocurrent, however, has no obvious counterpart in the 410-nm absorbance signal (M formation and decay).

Fig. 5 *C* shows the areas under the 1- and 6-ms components of R82Q, proportional to the charge moved during each photocurrent-producing transition (Trissl, 1990), plotted against pH, along with the pH dependence of the amplitude of the M intermediate. The M amplitude decreases with decreasing pH with a pK_a of 7.1 ± 0.1 ($n = 1$), mainly as a result of the transformation of purple membrane to blue membrane as D85 becomes protonated. Only when D85 is deprotonated in the unphotolyzed state can M be formed. The pK_a of 7.1 agrees well with values found for the pK_a of D85 from spectroscopic titrations of the blue membrane in R82Q and R82A (Brown et al., 1993; Balashov et al., 1993) and is shifted by 0.6 unit from the value of 6.5 found at 1 M NaCl (see above). The area of the 6-ms component shows a similar variation with pH.

In contrast, the variation of the 1-ms area with pH is more complex, but it could be well fitted with the equation

$$\text{Area} = C^*(1 + 10^{(pK_1 - pH)}) \times (1 + 10^{(pK_2 - pH)})^{-1},$$

which is simply the multiplication of two Henderson–Hasselbalch ($n = 1$) titration equations. The two pK_a values were calculated to be 7.1 ± 0.1 and 8.3 ± 0.2 respectively. The pK_a of 7.1 is interpreted as merely reflecting the processes modulating the amount of deprotonated D85 and thus the amount of M intermediate formed, as explained above. The second pK_a value reflects the titration of an unknown group whose protonation state controls the area of the 1-ms photocurrent. This is exactly the titration behavior that is expected from a photocurrent caused by the movement of a proton from the proton release group into solution or from an internal (or conformational) charge movement coupled to the action of the proton release group. We suggest that the 1-ms photocurrent is directly related to early proton release in R82Q, and its pK_a reflects the pK_a of the proton release group (E204) at 150 mM salt concentration. The photocurrent measurements thus provide an alternative method of performing titrations of groups involved in charge movements, proton release, or both. The observed pK_a of the proton release group measured with photocurrents at 150 mM salt (8.3) complements the value obtained above with dyes at high (1 M NaCl) salt concentration (7.5, closer to the intrinsic value of the pK_a).

We examined the involvement of protons as the source of the 1-ms photocurrent phase by measuring photocurrents in a gel incubated in D_2O while maintaining the salt and buffer concentration. Both the 1- and the 6-ms photocurrents greatly increase in lifetime, slowing to 3.5 and ~15 ms (Fig.

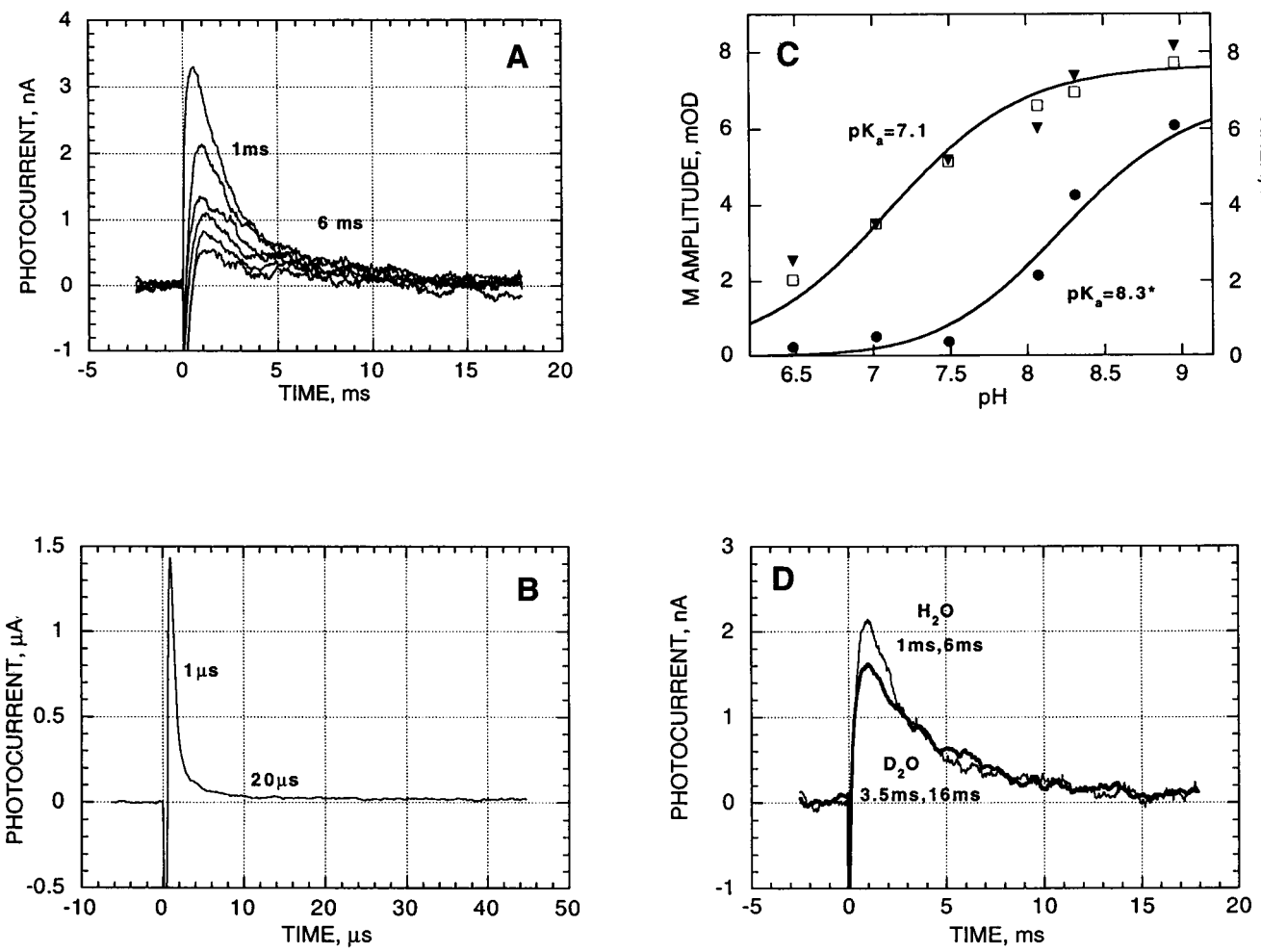


FIGURE 5 Photocurrents obtained from samples of R82Q oriented in a polyacrylamide gel (concentration 11 μM); photocurrent and absorbance traces are averages of 32–64 flashes. All samples are in 100 mM KCl/50 mM Tris. (*A*) Millisecond photocurrents, pH 6.5, 7.0, 7.5, 8.1, 8.3, 9.0 (increasing amplitude). Two positive photocurrent phases with 1- and 6-ms time constants are distinguishable. (*B*) Microsecond photocurrents, pH 9.0. Two positive photocurrent phases with 1- and 20-μs time constants are distinguishable and correlate well with M formation (not shown). (*C*) Variation with pH of M amplitude (□) and areas (amplitude × time constant) of the 1-ms (●) and 6 ms (▲) photocurrent phases. pK$_a$ values for the titrations are shown. The titration curve marked with an asterisk describes a process with a pK$_a$ of 8.3, scaled by a second process with a pK$_a$ of 7.1. See the explanation in Results. (*D*) Millisecond photocurrents in R82Q gels in H_2O (*thin trace*) and D_2O (*bold trace*), pH 8.3. Two positive photocurrents with the indicated decay time constants are observed in each sample.

5 *D*) respectively. This "D_2O effect" (Liu, 1990) strongly suggests the involvement of proton movement in the charge movements reflected by these photocurrents and provides further evidence that the 1-ms photocurrent reflects early proton release rather than a "conformational" charge movement such as the motion of a charged amino acid side chain.

These photocurrents were also measured in a sample incubated in 150 mM KCl/25% glycerol (data not shown); in this case, the 1-ms photocurrent slowed to ~3 ms, similar to the time constants of 3.5 ms measured for early proton release from R82Q (in 1 M NaCl/25% glycerol) using dyes (previous section). The higher salt concentration could not be used in the photocurrent measurements because the signal-to-noise ratio decreased. The kinetic data suggest that we have been able to isolate a photocurrent ($\tau \approx 1$ ms) that is due solely to proton release, which is distinct from other charge movements in the photocycle associated with the movement of the Schiff base proton to D85 ($\tau \approx 1$ or 20 μs); this is different from the WT photocycle in which these two charge movements are unresolved (Keszthelyi and Ormos, 1980; Liu, 1990; Trissl, 1990). Comparison of the areas under the microsecond and the 1-ms photocurrent phases of R82Q showed them to be approximately equal at pH 9.0 (~6 pA · s). Interestingly, both the pH-sensitive dye and the photocurrent measurements reveal the rate of early proton release of R82Q ($\tau \approx 1$ ms) to be significantly slower than that of the WT ($\tau \approx 85$ μs; see Discussion).

Proton release and uptake versus pH in R82K

As was reported earlier (Balashov et al., 1995), the order of proton release and uptake in R82K, at pH 7.4 and 150 mM

KCl (Fig. 6, *trace 1*), is the same as in the WT protein (proton release occurs before uptake). However, we find that the order of proton release and uptake can be reversed by lowering the pH. At pH 6.7 almost 60% of the protons are released early (Fig. 6, *trace 2*). In contrast to that of R82K, the WT bR shows a normal order of proton release before uptake at this pH (e.g., Govindjee et al., 1980). At pH 6.4 almost 80% of the protons are released after proton uptake (Fig. 6, *trace 3*). Thus the pK_a of the proton release group, when the pigment is in the M state, is ~6.6 (see Table 1). Photocurrent data were also obtained for R82K but were very difficult to interpret for several reasons. Between pH 4 and pH 9, photocurrents associated with the substantial amount of the 13-*cis* photocycle present in samples at these pHs complicate analysis (Balashov et al., 1995; Misra et al., 1996). Above pH 9, the proton release group in R82K becomes deprotonated in the unphotolyzed state (see Table 1 and Balashov et al., 1995). Proton release during the photocycle at this pH is either absent or too slow to be resolved with photocurrent methods, as the amplitudes of photocurrents are inversely proportional to their time constants (Trissl, 1990).

DISCUSSION

Role of R82 in the proton release process

A key observation of the present study is that the order of proton uptake and release in the R82Q mutant reverses when the pH is raised from pH 6.7 to pH 8.1. At higher pH it becomes almost similar to that in the WT at neutral pH: proton release precedes the uptake. This implies that R82 is not *X*, the normal proton release group in the WT, because we have found conditions (alkaline pH) under which early proton release can be observed even when R82 is absent. (Early proton release in the absence of R82 has also been reported in a double mutant, D212N/R82Q (Brown et al., 1995b).) We therefore suggest that in R82Q the early proton release (τ = 1 ms in 150 mM salt; 3.5 ms in 1 M salt/25% glycerol) occurs from the proton release group E204, which in this mutant can not deprotonate at pH 7 but can deprotonate at higher pH. In contrast, in E204Q, proton release is preceded by uptake even at high pH (Fig. 2), consistent with the proposed role of E204 as the usual proton release group (Scharnagl et al., 1994, 1995; Brown et al., 1995a; Sampogna and Honig, 1996). Thus the role of R82 in the proton release process can be discussed in terms of its effect on the pK_a of the proton release group in the unphotolyzed state and during the photocycle.

pK_a of the proton release group in R82Q and R82K

The pK_a of the proton release group in the M intermediate (when the Schiff base is deprotonated and D85 is protonated) can be inferred from the pH at which the order of light-induced proton release and uptake is reversed; this occurs when the pH of the medium is at the pK_a of E204. For the WT in 150 mM KCl this pK_a was found by Zimányi et al. (1992) to be 5.8. In R82Q (and R82A) the order of proton uptake and release is reversed near neutral pH (Fig. 1 *A*). Judging from the increase in the fraction of the early proton release phase as the pH is increased (Fig. 1 *A*), the pK_a of E204 (in the M state) is ~7.5 ± 0.3 in 1 M NaCl. An alternative method, based on the increase in the area under the 1-ms photocurrent, shows that the pK_a is 8.3 in 150 mM salt (Fig. 5 *C*). The different values can be attributed to surface potential effects, with the value in high salt being close to the intrinsic pK_a. Thus, replacement of R82 with a neutral residue (in R82Q) increases the pK_a of E204 (in M) by ~2.5 units (from 5.8 in the WT to ~8.3 in R82Q, both in 150 mM salt).

Replacement of R82 by another positively charged residue, lysine, partially lowers the pK_a of E204 (in M) from ~8.3 in R82Q to ~6.6 in R82K (in 150 mM salt). This value, however, is still approximately 0.8 pH unit higher than in the WT (5.8). Interestingly, a similar difference is seen in the pK_a of E204 in the respective unphotolyzed pigments, when D85 and the Schiff base are both protonated (4.9 in the WT versus 5.7 in R82K, a difference of ~0.8); see Table 1. Thus, this difference between pigments may be

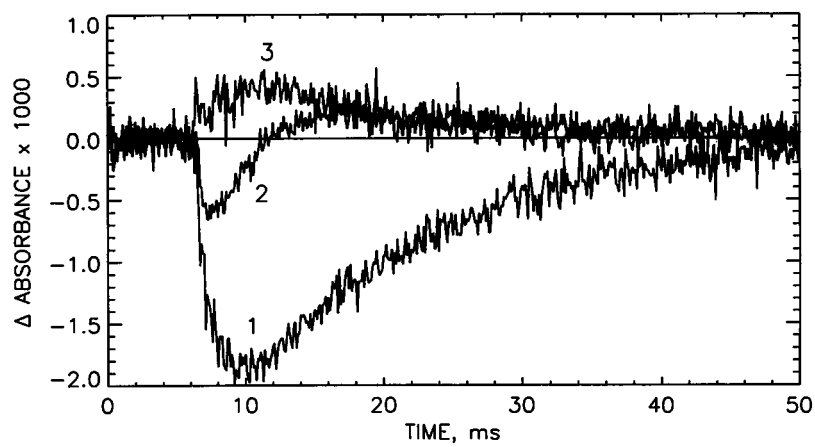


FIGURE 6 Flash-induced proton changes in R82K as measured with pyranine. Trace 1, pH 7.33, showing that proton release precedes uptake; trace 2, pH 6.65, showing ~60% early proton release and ~40% late proton release; trace 3, pH 6.4, showing ~20% early and ~80% late proton release. Similar kinetics were also observed with *p*-nitrophenol. 150 mM KCl, 20°C, $\lambda_{actinic}$ 532 nm, $\lambda_{monitoring}$ 460 nm. Dye traces have been normalized to same amount of M intermediate.

TABLE 1 pK_a values of the proton release group E204, and of D85, in different states of bR

Pigment	Salt	pK_a of E204 in Unphotolyzed Purple Membrane	pK_a of E204 in Unphotolyzed Blue Membrane	pK_a of E204 in M	Low pK_a of D85	High pK_a of D85
Protonation States of Key Groups		SBH^+ $D85^-$	SBH^+ D85H	SB D85H	SBH^+ E204H	SBH^+ E204
WT	150 mM	9.5*	4.9*	~5.8#	2.6*	7.2*
R82K	150 mM	8.0§	5.7§	~6.6¶	3.7§	6.0§
R82Q	150 mM	?	?	~8.3‖	7.2‖	?
R82Q	1 M	~8.0**	?	~7.5##	6.5**	?

SB, Schiff base.
*Balashov et al. (1993).
#Zimányi et al. (1992).
§Balashov et al. (1995).
¶Fig. 6.
‖Fig. 5.
**Fig. 3.
##Fig. 1.

due to the difference in the structure of the side chains of the two residues, lysine (in R82K) and arginine (in the WT), which has a similar quantitative effect on the pK_a of E204, both in the unphotolyzed state and during the photocycle, in M. Clearly, the guanidinium group of R82 plays an important role in maintaining the proper pK_a values of two of the key residues involved in proton transport, D85 and E204.

It is important to mention that R82 is only one of several residues and factors that control or influence the pK_a values of D85 and E204. According to Brown et al. (1995b), substitution of neutral residues for both R82 and D212, as in R82Q/D212N, lowers the pK_a of D85 in the unphotolyzed pigment from 7.2 in R82Q to ~1 in the double mutant (a value closer to that in the WT). Probably the pK_a of E204 is also restored to its near-normal value, because the normal order of proton release followed by uptake is observed in R82Q/D212N.

R82 could affect the pK_a of E204 by means of purely electrostatic interactions (Balashov et al., 1993; Sampogna et al., 1994). Recently it was suggested that the guanidinium group of R82 could move in the photocycle, aligning itself with D85 in the unphotolyzed state and with E204 during the photocycle, causing the latter to deprotonate and resulting in early proton release (Scharnagl et al., 1994, 1995; Brown et al., 1995a). Alternatively, inasmuch as it has been suggested that R82 is hydrogen bonded by water molecules to D85 and other residues (Cao et al., 1991; Zhou et al., 1993; Maeda et al. 1994; Humphrey et al., 1994), its mutation should affect the pK_a of D85 (Subramaniam et al., 1990; Otto et al., 1990; Miercke et al., 1991; Thorgeirsson et al., 1991; Balashov et al., 1993, 1995), alter the water structure in the proton channel, or both, which in turn could affect the pK_a of E204.

The pK_a of the proton release group in the unphotolyzed state in the WT has been inferred from the high pK_a transition in the pH titration of the fraction of blue membrane (protonated D85) and in the rate of dark adaptation; it is 9.5–9.7 in 150 mM KCl (Balashov et al., 1993, 1996). Similar measurements in the R82K mutant suggest that the pK_a of E204 is 8 in 150 mM KCl (see Table 1 and Balashov et al., 1995). In R82Q it is unclear what the pK_a of E204 is in the unphotolyzed state, because a clear second transition in the titration of the rate of dark adaptation is not observed (Richter et al., 1996). The pK_a of the proton release group in the unphotolyzed state, however, can also be estimated from pH-dependent changes in the kinetics of M formation. In the WT, M formation becomes clearly biphasic at alkaline pH levels. The fraction of M^f_{rise} (fast component of M formation) increases with a pK_a of ~9.2 in 150 mM KCl (Balashov et al., 1991), and a similar pK_a (~9.2 in 150 mM KCl) was observed by Kono et al. (1993) for the disappearance of a photocurrent component, which was ascribed to the light-induced deprotonation of the proton release group. This suggests that M^f_{rise} reflects the protonation state of the proton release group and that one could determine the pK_a of the latter by measuring the pK_a of M^f_{rise}. It should be mentioned that there is a small discrepancy between the pK_a of the proton release group as determined from the rate of dark adaptation (and the fraction of protonated D85) and from the fraction of M^f_{rise}; they are 9.5–9.7 and 9.2, respectively.

Similar to that in the WT but unlike in R82A, M formation in R82Q in 1 M NaCl/25% glycerol is clearly biphasic and pH dependent. In R82Q the pK_a of M^f_{rise} and thus the pK_a of E204 is ~8.0 ± 0.2 in 1 M NaCl (Fig. 3 *B*, *inset*). The difference between R82A and R82Q may be due to the fact that, although both alanine and glutamine are neutral residues, only the latter is capable of H-bonding. According to Humphrey et al. (1994), R82 is H-bonded by means of water molecules to E204. It is possible that Q82 is able to maintain the H-bonding to E204 and D85 that is reflected in the kinetics of the Schiff base deprotonation and M formation. These data thus suggest that R82 regulates the pK_a of

the proton release group E204 in the unphotolyzed state as well as in the M intermediate.

Origin of increased rate of Schiff base deprotonation

The increase in the fraction of M^f_{rise} with increasing pH and the subsequent deprotonation of E204 can be explained in terms of the four-state model proposed by Balashov et al. (1993, 1995, 1996), which suggests that D85 has two values of pK_a, depending on the protonation state of the proton release group E204. When the E204 is protonated, the pK_a of D85 is low, and when E204 is deprotonated (at high pH or in the M state of the photocycle after proton release), the pK_a of D85 is elevated (see the scheme in Introduction). This increase in the pK_a of D85 when E204 is deprotonated would result in an increased proton affinity for D85 and a reduction in the ΔpK_a between the Schiff base and D85. Both of these conditions would facilitate the transfer of the proton from the Schiff base to D85 during L → M transition and cause an increase in the fraction of M^f_{rise}. The pK_a of M^f_{rise} would therefore be indicative of the pK_a of E204 (see also Richter et al., 1996).

Amplitude of the light-induced proton signals

For a pigment to have a large light-induced proton signal, the proton release group should be fully protonated in the unphotolyzed state, and its pK_a should drop enough in M to allow complete deprotonation. In the WT, at neutral pH, the proton release group is fully protonated in the unphotolyzed state because its pK_a is high (~9.5), whereas in the M state its pK_a decreases by almost 4–5 units, allowing for full deprotonation (see Table 1); this results in a large (absorbance change of a pH-sensitive dye) proton release signal near neutral pH. In R82K and R82Q the pK_a of E204 in the unphotolyzed state is lower than in the WT, and it decreases by approximately 1 pH unit or less on formation of the M intermediate (Table 1). This small decrease in the pK_a of E204 during the photocycle at least partly explains the small amplitudes of the proton signals in R82Q and R82K. In these mutants at neutral pH, E204 is only partly protonated in the unphotolyzed state, and, as the medium pH is close to the pK_a of E204 in M, full light-induced deprotonation of what was initially protonated does not occur. Also, because the rate of proton uptake becomes closer to the rate of proton release, the amplitude of the proton signal is reduced. A similar explanation could be applied to other mutants also, e.g., Y57F, for which an inversion of the proton signal is seen at neutral pH.

In R82Q late proton release occurs from D85 during the decay of O

In the WT pigment, at low pH, proton uptake occurs after reisomerization of the chromophore, that is, after O formation, and late proton release is almost concurrent with O decay (Zimányi et al., 1992; Cao et al., 1993). In R82Q the sequence of proton uptake and release at neutral pH is similar to that seen in the WT pigment at low pH. However, in contrast to that in the WT (at low pH), in R82Q at neutral pH proton uptake occurs during the decay of the slow component of M and the formation of O, $\{M \Leftrightarrow N\} \rightarrow O$ transition (Fig. 3 *B*). This has also been reported for the L93T (Cao et al., 1993) and E204Q (Brown et al., 1995a) mutants. It is interesting to note that at higher pH the slow component of M decay becomes slower, as does the rate of proton uptake. Late proton release, whose time constant is similar to that of O decay in both R82Q and E204Q (also see Brown et al., 1995a), probably occurs directly from D85 on its deprotonation during the O → bR transition. Gerwert et al. (1990) have shown that, in the WT, D85 deprotonation tracks O decay, although in this case the proton probably goes to reprotonate E204, whereas we suggest a different fate for the proton in other circumstances (see below).

Differing pathways for proton release in bR

Table 1 summarizes different pK_a values found for E204 in this and previous studies for the WT, R82K, and R82Q. The values were obtained from spectroscopic titrations of D85 in the unphotolyzed states of the respective pigments as well as from titrations of the photocycle (M rise kinetics) and direct monitoring of proton release by dyes or photocurrents. The constant difference between the pK_a of E204 in the unphotolyzed state (the pK_a of E204 in the blue membrane) and the pK_a of E204 in M is evident for the WT and for R82K:

$$\Delta pK_{a(WT)} = 5.8 - 4.9 \approx \Delta pK_{a(R82K)} = 6.6 - 5.7,$$

reinforcing the idea that, despite the fact that the two situations are not completely analogous, the protonation of D85 has the effect of greatly lowering the pK_a of E204. The fact that these two values for the pK_a of E204 when D85 is protonated are not identical (e.g., 5.8 and 4.9; 6.6 and 5.7) does imply that other criteria, notably the protonation state of the Schiff base and the isomerization state of the pigment, also modulate the pK_a of the proton release group. Taken together, the values in Table 1 define those pH regions in which different pathways of proton release are utilized by the respective pigments.

A schematic representation of proton release, and the protonation status of some of the key residues in the extracellular domain of the proton channel under different pH conditions, is depicted in Fig. 7. It shows that early proton release occurs when the medium pH is below the pK_a of E204 in the unphotolyzed state and above its pK_a in the M state. Proton release is delayed at low pH (when E204 thermodynamically cannot deprotonate) or, analogously, in mutants in which E204 is replaced by a neutral residue (as in E204Q). Late proton release should also be evident in the WT at very high pH, where E204 is already deprotonated in

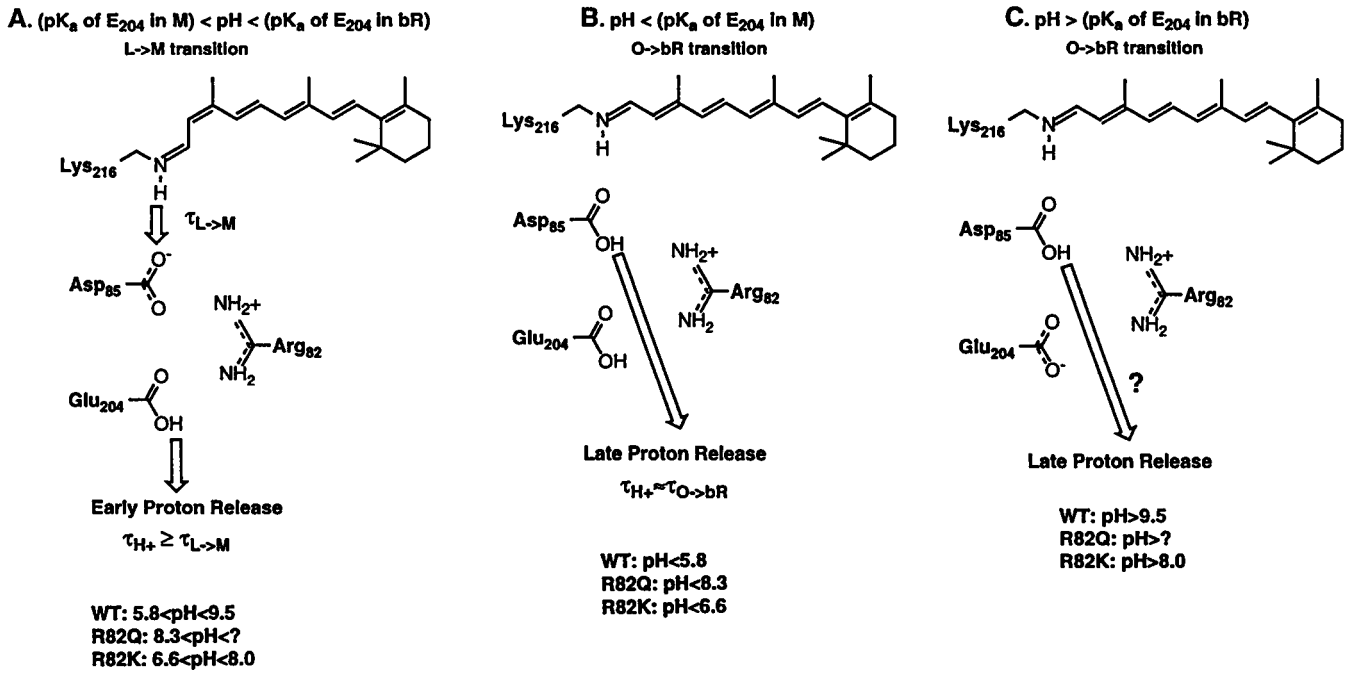


FIGURE 7 Schematic representation of early and late proton release and the protonation status of some of the key residues in the extracellular proton release pathway under different conditions. (*A*) Early proton release occurs from E204 during the L → M transition when the medium pH is lower than the pK$_a$ of E204 in the unphotolyzed state but higher than the pK$_a$ in M. (*B*) At low pH (when the bulk pH is lower than the pK$_a$ of E204 in M) late proton release occurs from D85 during the O → bR transition because E204 can not deprotonate. (*C*) At high pH E204 is deprotonated in the unphotolyzed state, and proton release should be delayed as in (*B*).

the unphotolyzed state of the pigment. The situations depicted in Fig. 7 *A* and *B* were described for the WT by Lanyi (1993); they are obviously also applicable (taking into account the alteration of the relevant pK$_a$ values) to various bR mutants. The situation depicted in Fig. 7 *C* has so far not been investigated experimentally; the simple experiment of monitoring proton release in the WT at high pH is difficult, chiefly because of the scarcity of pH-sensitive dyes that can operate reliably at strongly alkaline pH. Photocurrent measurements are impractical for monitoring slow charge movements, as the current's amplitude varies inversely with its time constant; for charge movements concurrent with the O → bR transition (tens of milliseconds or slower), the signals become indistinguishable from amplifier noise. Photovoltage measurements also have difficulty in quantifying proton release on such time scales, as current experimental systems tend to have discharge kinetics of the same order of magnitude as the kinetics of the O → bR transition.

A distinct photocurrent associated with early proton release

To date, studies of charge movements in bR and bR mutants have not been able to differentiate photoelectric signals that are due to the separate movements of the proton from the Schiff base to Asp-85 and those from the proton release group into solution, as these motions appear to occur concurrently with the L → M transition in the WT (Drachev et al., 1984; Liu, 1990; Heberle and Dencher, 1992; Alexiev et al., 1995). In R82Q an early proton release phase is evident at neutral pH (Fig. 1 *A*), but both the dye and the photocurrent kinetics suggest that it is decoupled from M formation, which has a time constant of ~1 μs. At alkaline pH, we are able to observe separate photocurrents in R82Q, which can be ascribed to M formation and to proton release, respectively, because of their correspondence, or lack thereof, to absorbance signals at 410 nm and to pH-sensitive dye signals. The photocurrent phase with a time constant of ~1 ms in water slows to 3.5 ms in D_2O, suggesting that it arises from proton movement. In addition, in conditions similar to those in which the dye experiments were performed (25% glycerol), this photocurrent phase slows down to ~3 ms, agreeing well with the time constant of early proton release as measured by the dyes. Finally, the titration behavior of the photocurrent agrees well with the suggestion that the photocurrent is caused by proton release, as it disappears at pH values at which proton dissociation from *X* becomes thermodynamically unfeasible.

Correlation of the rate of early proton release with the pK$_a$ of the proton release group: what is the rate limiting step in proton release?

In WT bR at neutral pH, early proton release is coincident with M formation: both occur with a time constant of ~85 μs (Drachev et al., 1984; Liu, 1990; Heberle and Dencher,

1992; Scherrer et al., 1992). The dye and photocurrent kinetics suggest, however, that early proton release occurs more slowly in R82Q than in the WT, with a time constant of 1 ms (3 ms in 25% glycerol). It seems reasonable to propose that this slowdown is at least partially due to the elevated pK_a of E204 (in M) in R82Q relative to the WT (see Table 1). The simple expression for the dissociation constant of a proton:

$$K_d \approx 2 \times 10^{(10-pK)} s^{-1}$$

based on a diffusion-controlled proton association constant for aqueous media (Gutman and Nachliel, 1995), gives a time constant of several microseconds for proton release from the WT (intrinsic pK_a of the proton release group of $\approx$5) but would predict a proton release time constant of several milliseconds, 3 orders of magnitude greater, for R82Q (intrinsic pK_a of $\approx$7.5). Indeed a much longer proton release time, presumably from E204, is what is observed in R82Q. In the WT, the rate of protonation of D85 in M (or changes in protein conformation (Subramaniam et al., 1993)), rather than the equilibration of the proton between E204 and the bulk solution, could be the rate-limiting step in proton release. In R82Q, however, the relatively higher pK_a of the proton release group during M would limit the rate of "early" proton release even though M is formed rapidly, because the proton takes much longer to be released from E204 into solution.

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