Glutamate-194 to Cysteine Mutation Inhibits Fast Light-Induced Proton Release in Bacteriorhodopsin[†]

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ABSTRACT: Substitution of glutamic acid-194, a residue on the extracellular surface of bacteriorhodopsin, with a cysteine inhibits the fast light-induced proton release that normally is coupled with the deprotonation of the Schiff base during the L to M transition. Proton release in this mutant occurs at the very end of the photocycle and coincides with deprotonation of the primary proton acceptor, Asp-85, during the O to bR transition. The E194C mutation also results in a slowing down of the photocycle by about 1 order of magnitude as compared to the wild type and produces a strong effect on the pH dependence of dark adaptation that is interpreted as a drastic reduction or elimination of the coupling between the primary proton acceptor Asp-85 and the proton release group. These data indicate that Glu-194 is a critical component of the proton release complex in bacteriorhodopsin.

Light-induced proton transport in bacteriorhodopsin (Lanyi, 1993; Ebrey, 1993; Ottolenghi & Sheves, 1995) starts with the light reaction bR → K which leads to a change of the proton affinity of the Schiff base and subsequent transfer of a proton from the Schiff base to the counterion, Asp-85, during the L to M transition. In the WT[1] under normal conditions, the L → M transition is accompanied by proton release to the extracellular surface of the membrane. The intramolecular proton movement has been studied with photovoltage and photocurrent measurements (Drachev et al., 1981; Keszthelyi & Ormos, 1983; Liu et al., 1990). Release of the proton to the surface was detected with pH sensitive dyes bound to the surface of the membrane (Heberle & Dencher, 1992; Cao et al., 1995; Alexiev et al., 1995). It takes place with $\tau_e \approx 80$ μs in the WT. Appearance of the released protons in the bulk solvent occurs with ca. 0.8 ms delay, due to a slow diffusion of protons from the surface into the bulk in the absence of buffers (Drachev et al., 1984; Heberle & Dencher, 1992; Cao et al., 1995; Alexiev et al., 1995). An interesting aspect of the proton release process is that the primary proton acceptor, Asp-85, remains protonated until the end of the photocycle (Braiman et al., 1988; Souvignier & Gerwert, 1992) so the proton which is released during the L → M transition must come from some other group, called X (Siebert et al., 1982; Zimányi et al., 1992; Lanyi, 1993; Ebrey, 1993).

At low pH, the formation of the M intermediate and protonation of Asp-85 is not accompanied by fast proton release. Instead, release occurs in the last step of the photocycle, upon deprotonation of Asp-85 during the O → bR transition (Dencher & Wilms, 1975; Zimányi et al., 1992), after proton uptake takes place during the preceding N → O transition. The switch between the two types of proton release, early (fast), which correlates with the L → M transition, and late (slow), which correlates with the O → bR transition, depends on the pK_a of the proton release group X. The pK_a of the proton release group in the WT is 5.8 in the M state (Zimányi et al., 1992). At low pH (pH < 5) this group is unable to release a proton in the M state so proton release occurs upon the deprotonation of Asp-85 during reformation of the initial state of bR.

The pK_a of the proton release group in the initial state of the pigment has not yet been determined directly by titration of light-induced proton release with pH sensitive dyes; however several related phenomena such as the pH dependence of the photocurrent accompanying M formation (Kono et al., 1993), the pH dependence of the rate constant of M formation, and the pH dependence of dark adaptation and titration of Asp-85 (Balashov et al., 1993; 1996) indicate that the pK_a of this group in the initial state of WT is ca. 9.5 in 150 mM KCl. Thus the most probable mechanism for the fast proton release is that photoisomerization of the chromophore in bR → K photoreaction and collapse of the Schiff base−Asp-85 ion pair during the L → M transition leads to a decrease in the pK_a of the proton release group, so that it can deprotonate in the M state at neutral pH (Lanyi, 1993; Ebrey, 1993; Balashov et al., 1993, 1995, 1996; Govindjee et al., 1996; Scharnagl et al., 1995; Sampogna & Honig, 1996).

We have found that in the WT and in several mutants the thermal isomerization of the chromophore is catalyzed by the protonation of Asp-85 (Balashov et al., 1993, 1995, 1996) and that titration of Asp-85 does not behave as a simple protonation−deprotonation reaction of an isolated residue. Instead, the titration can be described by the strong coupling of the pK_a of Asp-85 with the protonation state of another residue, X′, so that deprotonation of X′ causes an increase in the pK_a of Asp-85 by 5 pK units. Similarly, the protonation of Asp-85 causes a decrease in the pK_a of X′ by

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[1] Abbreviations: bR, bacteriorhodopsin; WT, wild type; DA, dark adaptation.

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about 5 p*K* units in the initial state of the pigment. We have suggested that the group X′ is in fact the proton release group X at the initial state of the pigment (Balashov et al., 1993, 1995).

The identity of the proton release group and the mechanism of coupling between this group and the chromophore is of great interest. Several mutations strongly affect both light-induced proton release and the pK_as of Asp-85, X′, and X. R82A and R82Q mutants show very slow proton release that takes place after proton uptake at neutral pH. However, at higher pH a faster proton release component appears (Govindjee et al., 1996). This observation indicated that although Arg-82 itself is probably not the proton release group, it can control the pK_a of the group or can be viewed as part of a cluster of residues involved in proton release. Theoretical calculations (Scharnagl et al., 1995; Sampogna & Honig, 1996) and experiments with E204Q and E204D mutants (Brown et al., 1995; Richter et al., 1996) have led to the suggestion that Glu-204 is the proton release group.

In the present paper we investigated the effect of a mutation of glutamic acid-194 on light-induced proton release and the pK_a's of Asp-85 and X′, as inferred from the pH dependence of dark adaptation and titration of Asp-85 (the purple to blue transition). In the recent structure of bR (Grigorieff et al., 1996), Glu-194 is in the loose turn terminating helix F; it may form a salt bridge with Arg-134 which is buried near the extracellular surface on helix E. We find that substitution of Glu-194 with cysteine completely abolishes fast light-induced proton release at neutral pH, similar to the effect of the E204Q mutation. The pH dependence of the rate constant of dark adaptation in E194C mutant indicates that the coupling between the proton release group and Asp-85 is lost or greatly decreased. These results imply that Glu-194 is a key part of the proton release complex in bR.

MATERIALS AND METHODS

The construction and expression of the E194C mutant in *Halobacterium salinarium* was done as described for the R82A mutant (Balashov et al., 1993). The absorption spectra were recorded on a Cary-Aviv-14DS spectrophotometer (Aviv Associates, Lakewood, NJ). Flash photolysis experiments were done as described previously (Govindjee et al., 1990). The pH sensitive dye pyranine, at a concentration of 25 μM, was used to measure the light-induced pH changes in the suspension. The pathlength of the cuvette was 5 mm. In order to obtain the light-induced absorption changes of the dye, the trace in the absence of the dye was subtracted from the trace taken in the presence of the dye. All measurements were done at 20 °C in suspensions of membranes. Acrylamide gels that prevent aggregation at low pH could not be used with the cysteine mutant because, when incorporated into gels, ammonium persulfate and TEMED treatment causes a modification of the pigment that resulted in a 20 nm blue shift in its absorption spectrum. A reducing agent, dithiothreitol (1 mM), which prevents oxidation of cysteine residues, was added to suspensions of E194C in some long dark adaptation and titration experiments; it did not noticeably affect the absorption spectrum. A mixture of six pH buffers (citric acid, Mes, Hepes, Mops, Ches, Caps) was used to stabilize the pH between 11 and 2 for the dark adaptation experiments. The optical density of the samples for flash-induced absorption changes measurements was 0.3−0.4.

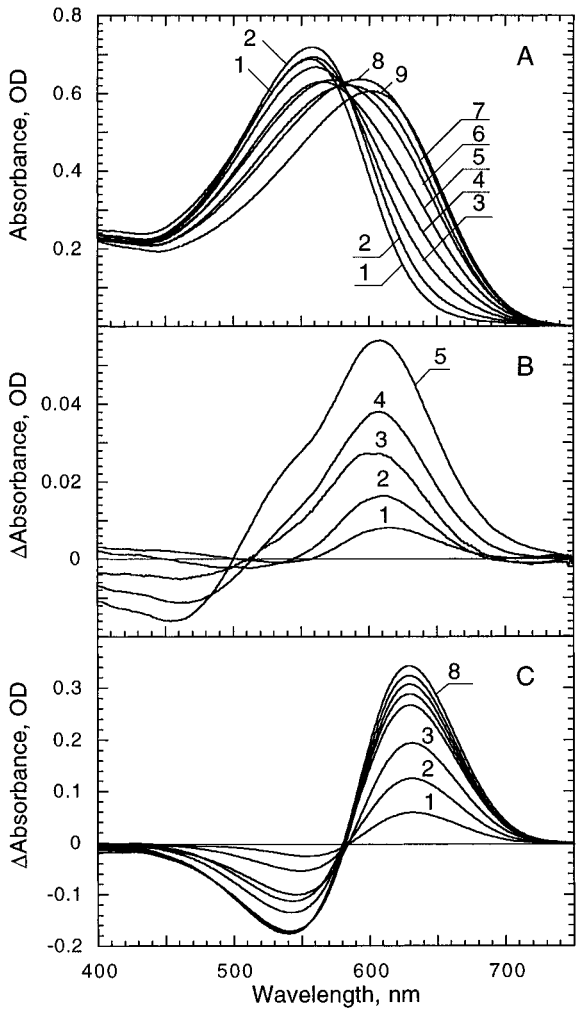

FIGURE 1: (A) Absorption spectra of dark-adapted E194C at pH: 6.8, 4.4, 3.4, 3.0, 2.8, 2.6, 2.2, 1.5, 0.8, curves 1−9, respectively. (B) Difference spectra of E194C at pH_i minus pH 6.8 where pH_i is equal to 6.3, 5.9, 5.5, 5.1, 4.4. (curves 1−5, respectively). (C) Difference spectra of E194C at pH_i minus pH 4.4, where pH_i is equal to 3.4, 3.0, 2.8, 2.6, 2.4, 2.0, 1.8, 1.5, and 0.8, curves 1−8, respectively, 150 mM KCl.

RESULTS

In order to characterize the effect of the Glu-194 → Cys mutation on the function of bR, we studied the pH dependence of spectral changes associated with the protonation of Asp-85 and with the formation of a species called P480; the pH dependence of the rate of thermal isomerization, which depends on the coupling of Asp-85 and the proton release group; the kinetics of late photocycle intermediates; and the kinetics of light-induced pH changes.

pH Dependence of the Absorption Spectra: Purple to Blue Transitions and Formation of P480-like Species in E194C. Upon decreasing the pH, the absorption maximum of the dark-adapted E194C pigment shifts from 556 nm at pH 6.8 to 604 nm at pH 0.8 (Figure 1 A) due to the "purple to blue" transition associated with protonation of Asp-85. The plot of absorbance changes at 660 nm (wavelength where the blue species predominantly absorbs) versus pH reveals a major (91%) transition with pK_a 3.0 and a minor (9%) transition

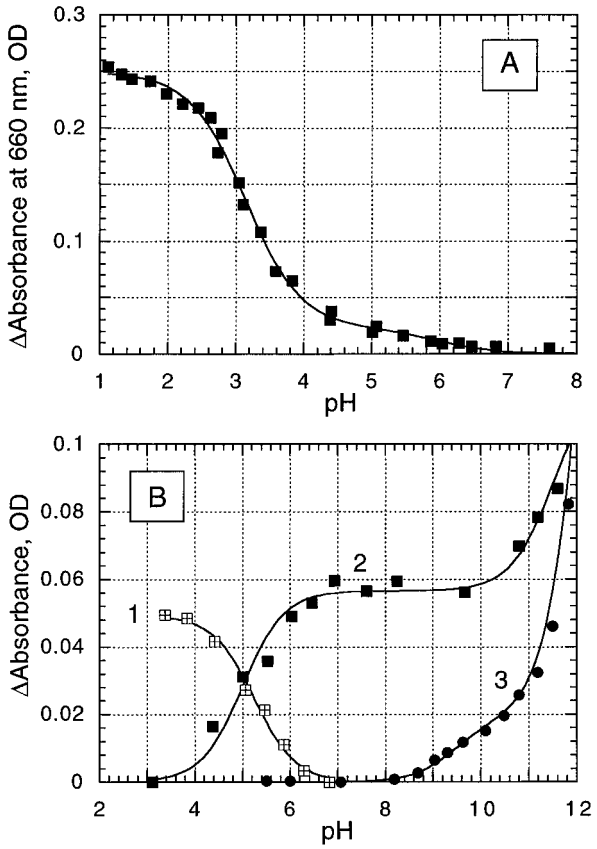

FIGURE 2: (A) pH dependence of absorption changes due to the purple-to-blue transition(s) measured at 660 nm; the line represents a two component fit, pK_a 3.0 (91%) and pK_a 5.8 (9%). (B) pH dependence of absorption changes due to formation of species absorbing at 460−480 nm (P480 species) measured in: 1, E194C at 575 nm; the curve is a one component fit with pK_a 5.2; 2, E194C at 460 nm; line is fit with pK_a 5.2 and 12.5. 3, WT at 460 nm, the curve is a two-component fit with pK_a 9.2 and 12.2. 150 mM KCl.

with pK_a 5.8 (Figure 2 A). The absorption changes are different in these two transitions (Figure 1B,C). The minor transition with pK_a 5.8 is characterized by a maximum at 610 nm in the difference spectrum (Figure 1B), while the major transition with pK_a 3 has absorption maximum at 630 nm and an isosbestic point at 580 nm (Figure 1C). The pK_a of the purple to blue transition in the WT is 2.6 (major component). At high pH a second component with pK_a ca. 9.5 was also detected (Balashov et al., 1996) which is due to coupling of the pK_a's of Asp-85 and the proton release group.

The isosbestic point is absent in the difference spectra taken between pH 6.3 and 4.4 (Figure 1B), indicating that two transitions take place at these pHs. The purple to blue transition with pK_a 5.8 overlaps with another transition that results in an increase of absorbance around 550−560 nm (see Figure 1A, curve 2, and Figure 1B, curve 5) and a decrease of absorbance at 460 nm upon decreasing the pH. The latter transition can be interpreted as transformation of a species absorbing at 460−480 nm into the purple form of the pigment. The pK_a of this transition, determined from the absorption changes at 575 nm (wavelength close to isosbestic point of the purple to blue transition) or at 460 nm, is 5.2 (Figure 2B, curves 1, 2). We will refer to the species absorbing around 460−480 nm as the P480-like species because it resembles P480, a species seen in the WT at high pH (Balashov et al., 1991). In the WT, partial transformation of the purple pigment into P480 occurs at much higher pH (pK_a ca. 9.2−9.5) than in E194C (pK_a ca. 5.2) (Figure 2B, curve 3; see also Balashov et al., 1991; Govindjee et al., 1997). Partial transformation of the pigment into P480 seems to indicate the pK_a of the proton release group in the ground state of the pigment (Govindjee et al., 1997). Formation of P480 in E194C at low pH suggests that the pK_a of the proton release group in the ground (unphotolyzed) state of this mutant may be very low (pK_a ca. 5.2) and so the group would be already deprotonated at neutral pH.

pH dependence of the rate constant of thermal all-trans ⇌ 13-cis isomerization of the chromophore (dark adaptation) may provide independent information on the pK_a of the proton relese group and its coupling to the primary proton acceptor Asp-85 (Balashov et al., 1996). In order to utilize this approach we examined the light and dark adaptation of E194C. Illumination of dark-adapted E194C at pH 6.8 causes a shift of the absorption maximum from 556 to 565 nm and an increase in maximum absorbance. The difference spectrum produced by light adaptation is similar to that in the WT. At pH > 5 light adaptation, along with the transformation of 13-*cis* pigment to *all-trans* pigment, causes an increase in the fraction of a species with an absorption maximum at 460 nm in the difference spectrum (P480 like species) by about 5% as follows from the absorption changes during dark adaptation (Figure 3). The increase in the fraction of this species upon light adaptation is observed presumably because it is formed from *all-trans*-bR. In the WT, the analogous light-induced formation and relaxation of the species called P480 is observed only at pH > 9 (Govindjee et al., 1997).

Dark adaptation of the E194C pigment is very slow at neutral pH (life time of main component is 63 h at 20 °C compared to 3 h in WT). Two different transitions are observed during first period of dark adaptation in the E194C mutant. The difference absorption spectra indicate that, besides the transformation of *all-trans*-bR back to 13-*cis*-bR, another minor and faster transformation takes place (Figure 3A). This process occurs during first 4 h of dark adaptation (at pH 6.8) and overlaps with thermal isomerization from *all-trans* to 13-*cis*-bR which occurs on a much longer time scale at pH 6.8 (Figure 3B). The difference spectrum of this transition is shown in Figure 3C, curve 3. It indicates that this transition involves transformation of a species absorbing at 460−480 nm to a species absorbing at 550 nm which is presumably the 13-*cis* form of the pigment. About 5% of the total pigment undergoes this transformation. The kinetics of this process can be followed at 530 nm, an isosbestic point between the *all-trans* and 13-*cis* forms of the pigment; its time constant is 62 min at pH 6.8. The contribution of this process is less at longer wavelengths (620−630 nm), where the kinetics of "normal" dark adaptation, the transformation of the *all-trans* into the 13-*cis* form of the pigment, can be monitored. After 4 h of dark adaptation, the transformation of the P480-like species is complete and further absorption changes are due exclusively to the transformation of *all-trans* to 13-*cis* form of the pigment (Figure 3B).

Kinetics of all-trans → 13-cis thermal isomerization determined from absorption changes at 620 nm are biphasic in E194C. At pH 6.8 the fast phase has a rate constant of 4.9×10^{-5} s^{-1} (τ_e = 5.7 h) with a relative amplitude of

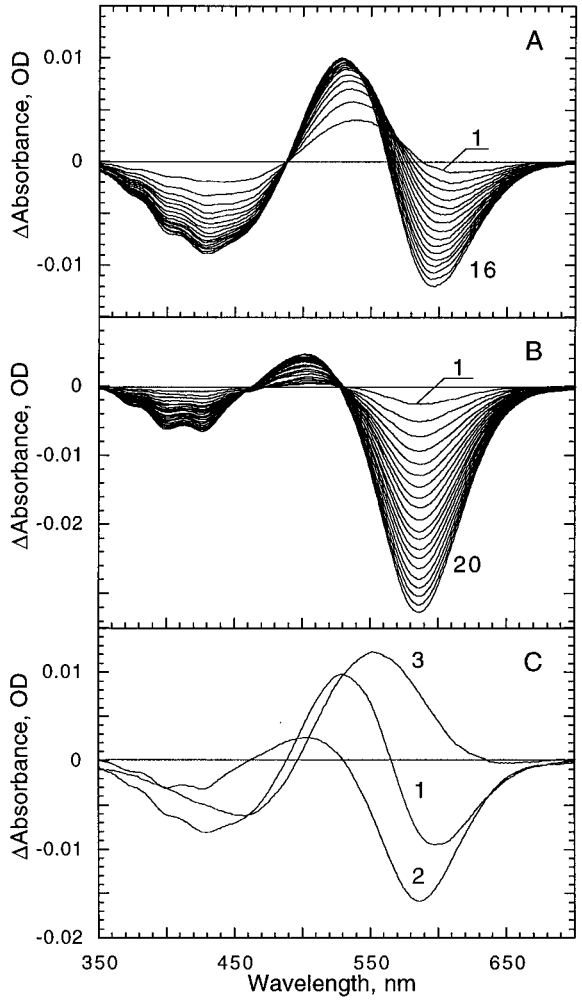

FIGURE 3: Absorption changes observed during dark adaptation of a suspension of E194C membranes in 75 mM K_2SO_4 at pH 6.8, 20 °C. (A) Difference spectra of dark-adapted minus light-adapted taken every 15 min during first 4 h of DA: 1, 15 min of DA; 16, 4 h of DA. (B) Difference spectra of dark-adapted minus spectrum after 4 h of DA taken between 5th and 24th hour of DA with 1 h interval: 1, 5 h of DA minus 4 h of DA; 20, 24 h of DA minus 4 h of DA. (C) Deconvolution of the absorbance changes after 4 h of DA into two components: 1, difference spectrum of 4 h in the dark-minus light-adapted, same as curve 16 in panel A. 2, difference spectrum due to transformation of *all-trans* pigment to 13-*cis* pigment during first 4 h of dark adaptation; 3, difference spectrum obtained as a difference between curve 1 and 2. It shows a minimum at 460 nm and a maximum at 550 nm due to conversion of the P480-like species into the purple form of the pigment.

about 8%. Importantly, the rate constant of the major component (92%) is much slower ($k_{da} = 4.5 \times 10^{-6}$ s^{-1} or $\tau_e = 63$ h). For comparison in the WT at this pH $k_{da} = 9 \times 10^{-5}$ s^{-1}.

The pH dependence of the rate constant of *all-trans* ⇌ 13-*cis* thermal isomerization in E194C mutant is shown in Figure 4A (the rate constant of the major component is plotted). Upon the pH being decreased, the rate constant becomes faster so that at pH 5 the rate constant in E194C is approximately the same as in the WT. At pH 2 the rate constant is 4 orders of magnitude faster than at pH 6.8 (Figure 4). Unlike for the WT [Figure 4B; see also Balashov et al. (1996)], pH dependence of the rate constant does not show a clear plateau nor a second transition at higher pHs. Instead, the pH dependence of the rate constant in E194C can be fitted with a single component with pK_a 2.7 (Figure

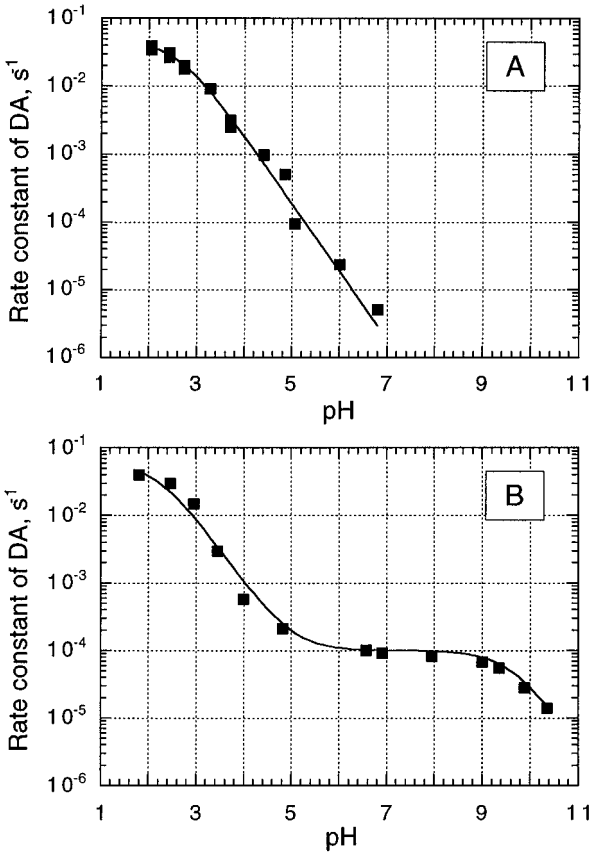

FIGURE 4: pH dependence of the rate constant of dark adaptation at 20 °C for suspensions of (A) E194C in 75 mM K_2SO_4; pH dependence in E194C is fitted as, $k_{da}(pH) = k°_{da}f(pH)$ (Balashov et al., 1993), where $k°_{da} = 0.045$ s^{-1} is the rate constant of dark adaptation when Asp-85 is protonated and the pigment is in the blue membrane, and f(pH) is the fraction of protonated Asp-85 described for the case of E194C by Henderson−Hasselbalch equation $f(pH) = (1 + 10^{(pH-pK_a)})^{-1}$; p$K_a$ 2.7; (B) WT in 75 mM K_2SO_4. The line is the fit with the model of two interacting residues, D85 and X′ with pK_a's 2.6 and 9.7 (Balashov et al., 1993, 1996).

4A). This compares well with the value of 3 found for the pK_a of the purple to blue transition (Figure 2).

The minor transition with pK_a 5.8 which is present in the titration of blue membrane at 660 nm (Figure 2A,B) is not seen in the pH dependence of dark adaptation shown in Figure 4A. This indicates that the component with pK_a 5.8 does not belong to the main fraction of the pigment but rather originates from a minor (8−9%) fraction having a higher pK_a for Asp-85 (5.8 versus 3). Apparently, the faster component of dark adaptation ($\tau_{da} = 5.7$ h at pH 6.8) is associated with this fraction of the pigment.

Light-Induced Photointermediates, Proton Release, and Uptake in E194C. Light-induced absorption changes at 410, 580, and 680 nm at pH 7.0 for E194C are depicted in Figure 5A. The initial pigment recovery measured at 580 nm at neutral pH is 5−7 times slower than in the WT. The lifetime of the O intermediate in E194C is longer than in the WT but shorter than in the E204Q mutant. M formation occurs with a time constant of 70 ± 5 μs at 20 °C. Unlike in the WT, there is no discernable pH dependence of the time constant for M rise in E194C between pH 4.5 and 10.5 (Figure 6).

The pyranine signal reflecting light-induced proton changes at pH 6.8 in E194C is shown in Figure 5B,C. In the E194C mutant, light-induced proton uptake (with time constant 21

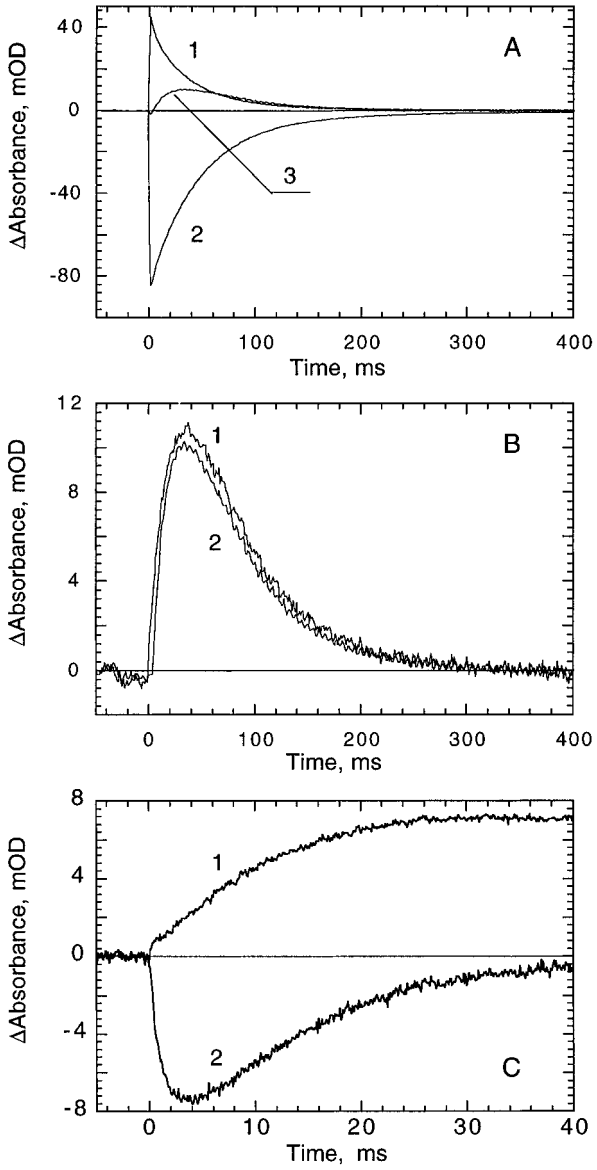

FIGURE 5: A. Kinetics of light-induced absorption changes at 410 (1), 580 (2), and 680 nm (3) in E194C at pH 7.0. B. Light-induced absorption changes: 1, of pyranine at 458 nm due to light-induced pH changes of E194C (increase of absorbance corresponds to proton uptake, decrease of absorbance corresponds to proton release); 2, of the E194C pigment at 680 nm due to the formation and decay of the O intermediate. 20 °C, 150 mM KCl. pH 6.8. (C) Comparison of pyranine signal in E194C (1) and wild-type (2), pH 6.8.

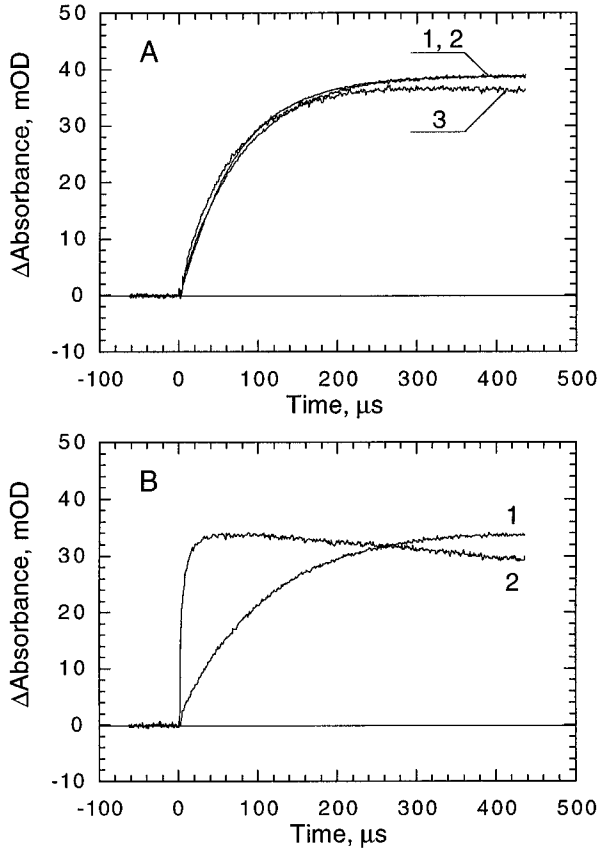

FIGURE 6: Comparison of kinetics of formation of the M intermediate (monitored at 410 nm) in E194C mutant and WT at different pHs. (A) E194C. 1, pH 4.5, 2, pH 6.8; 3, pH 10.5. The rise time of the M intermediate is 69 ± 1 μs at pH 4.5, 73 ± 2 μs at pH 6.8, and 65 ± 4 μs at pH 10.5. At pH 6.8 and pH 10.5 a small (8% of the total amplitude) fast (3−5 μs) component is also present. It may originate from the minor fraction of the pigment (9%) having pK_a of the purple-to-blue transition of 5.8. (B) WT. 1, pH 6.8; 2, 10.5. 20 °C, 150 mM KCl.

ms) is observed first, followed by proton release with a time constant of 60 ms. The proton uptake and release almost coincide with the formation and decay of the O intermediate (Figure 5B, curve 2). No fast proton release signal was observed at pH 6.8 (Figure 5C, curve 1). This is very different from the wild-type where proton release occurs with time constant less than 1.5 ms, and proton uptake takes place with time constant 13 ms (Figure 5C, curve 2). From the data shown in Figure 5 we conclude that fast proton release is inhibited in the E194C mutant at neutral pH. Only slow proton release takes place.

DISCUSSION

Glu-194 is a part of the proton release complex. The E194C mutant shows features similar to that of the E204Q mutant (Brown et al., 1995; Richter et al., 1996): inhibition of the light-induced fast proton release (Figure 5), a slow photocycle compared to the WT (Figure 5), a single-component titration curve for the pH dependence of dark adaptation and thus of Asp-85 (Figure 4), and lack of pH dependence of M formation (Figure 6). This suggests that Glu-194, like Glu-204, is part of the proton release complex. There are two possible mechanisms for the involvement of Glu-194 in the process of proton release, direct and indirect. In the first case Glu-194 may directly participate in proton transfer and share a proton with other groups. The second possibility is that Glu-194 controls the pK_a of the proton release group (Glu-204), through control of the conformation of this group, or its environment.

The pH dependence of dark adaptation and thus of the protonation state of Asp-85 can be fitted reasonably well, with one component indicating that the strong coupling between Asp-85 and the proton release group seen in the WT is absent or dramatically weakened in E194C. The absence of coupling of Asp-85 with the proton release group correlates with the absence of a fast component of proton release in E194C mutant. Fast proton release is inhibited in two situations. (i) The pK_a of the proton release group is high in the ground state and does not drop sufficiently during the photocycle (in M state); then no fast proton release is observed at neutral pH. This is precisely what occurs in

the R82Q mutant (Govindjee et al., 1996). (ii) Another possibility is that the pK_a of the normal proton release group is low in the ground state (<6) of the mutant, and being deprotonated, it cannot serve as the proton release group. It is not obvious from the pH dependence of dark adaptation in E194C what the pK_a of the proton release group in the ground state is, because there is no clear second transition which can be attributed to the deprotonation of the proton release group as in WT (Balashov et al., 1993, 1995, 1996; Richter et al., 1996). However, the pH dependence of dark adaptation indicates the lack of coupling between this group and Asp-85.

In E194C, formation of the species with an absorbance maximum at 460−480 nm has a pK_a around 5.2. In the WT, a similar transition that results in partial transformation of the pigment into P480 occurs with pK_a 9−9.5 (Balashov et al., 1991; Govindjee et al., 1997). We have previously shown that the partial transformation of bR into P480 may be attributed to deprotonation of the proton release group in the initial state of the pigment (Govindjee et al., 1997). If this holds true for the E194C mutant, then we can conclude that the pK_a of the proton release group in the ground state of this mutant may be very low, around 5 (versus 9.5 in the WT). The pK_a does not change much upon protonation of Asp-85 (since the pH dependence of dark adaptation can be fitted with a single component).

The kinetics of the formation of the M intermediate in E194C is practically pH independent between 4.5 and 10.5. This is similar to E204Q mutant (Richter et al., 1996) but is different from the WT, which shows a dramatic increase in the rate of M formation between pH 9 and 10 from ca. 85 μs at neutral pH to 0.3 μs (60%) and 6 μs (40%) at high pH (Kalisky et al., 1981; Liu, 1990; Balashov et al., 1991). The increase in the rate constant of M formation has been attributed to the deprotonation of the proton release group in the ground state which, due to coupling, increases the proton affinity of Asp-85 (Balashov et al., 1995; Richter et al., 1996). The lack of the increase in the rate constant of M rise in E194C at high pH is in agreement with the conclusion that the coupling between Asp-85 and the proton release group is absent or dramatically decreased in E194C and/or that the pK_a of the proton release group is greatly decreased in the ground state.

Thus, the data presented indicate that, in eliminating Glu-194, we have removed a crucial portion of the normal proton release machinery, since light-induced fast proton release is no longer observed. Glu-194 in native bR may serve as a part of the proton release group, or as a structural constraint that keeps the pK_a of the proton release group high in the ground state. A salt bridge with Arg-134 may serve this purpose.

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