The Two pK_a's of Aspartate-85 and Control of Thermal Isomerization and Proton Release in the Arginine-82 to Lysine Mutant of Bacteriorhodopsin†

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ABSTRACT: To explore the role of Arg82 in the catalysis of proton transfer in bacteriorhodopsin, we replaced Arg82 with Lys, which is also positively charged at neutral pH but has an intrinsic pK_a of about 1.7 pH units lower than that of Arg. In the R82K mutant expressed in *Halobacterium salinarium,* we found the following: (1) The pK_a of the purple-to-blue transition at low pH (which reflects the pK_a of Asp85) is 3.6 ± 0.1. At high pH a second inflection in the blue-to-purple transition with pK_a = 8.0 is found. The complex titration behavior of Asp85 indicates that the pK_a of Asp85 depends on the protonation state of another amino acid residue, X′, which has a pK_a = 8.0 in R82K. The fit of the experimental data to a model of two interacting residues shows that deprotonation of X′ at high pH causes a shift in the pK_a of Asp85 from 3.7 to 6.0. In turn, protonation of Asp85 decreases the pK_a of X′ by 2.3 pH units. This suggests that X′ can release a proton upon formation of the M intermediate and the concomitant protonation of Asp85 in the photocycle. (2) The rate constant of dark adaptation, k_{da}, is proportional to the fraction of blue membrane between pH 2 and 10, indicating that thermal isomerization proceeds through the transient protonation of Asp85. The pH dependence of k_{da} shows that two groups with pK_{a1} = 3.9 and pK_{a2} = 8.0 control the rate of dark adaptation in R82K. The 1.7 pH unit shift in pK_{a2} in R82K compared to the wild type (WT) (pK_{a2} = 9.7) supports the hypothesis that X′ is Arg82 in WT and Lys82 in R82K (or at least that these groups are the principal part of a cluster of residues that constitute X′). (3) Under steady state illumination, the efficiency of proton transport in R82K incorporated in phosphatidylcholine vesicles is at least 40% of that in the WT. A flash-induced transient signal of the pH-sensitive dye pyranine is similar to that in the WT (proton release precedes uptake), but the amplitude is small in R82K (about 15% of that found in the WT), indicating that only a small fraction of protons is released fast in R82K. This supports the suggestions that Arg82 is associated with the proton release pathway (acts as a proton release group or part of a proton release complex) and that Lys cannot efficiently substitute for Arg in this process. (4) R82K shows a pH-independent rate of M formation, lack of accumulation of the O intermediate, and fast decay of the bathoproduct of the 13-cis pigment, K^c (0.2 ms). At low and neutral pH, R82K contains a significant fraction of 13-*cis*-bR in the light-adapted state since *trans*-bR is converted back into 13-*cis*-bR under illumination at pH < 9. The pH dependence of light adaptation results in a pH-dependent yield of the M intermediate (pK_a = 8.0). Two other reversible transitions with similar pK_a's are observed: a 2 nm red shift of the chromophore absorption band and a red shift of the absorption band of a Trp residue. (5) Lys82 is accessible to modification by acetic anhydride. Acetylation of Lys82 shifts the pK_a of Asp85 to 6.5, which agrees with the prediction of our model for the pK_a of Asp85 when X′ is deprotonated.

Arg82 is an important residue in the retinal binding pocket of bacteriorhodospin (bR).[1] It stabilizes the ionized state of Asp85, which acts as a proton acceptor and part of a counterion to the Schiff base [discussed in the following reviews: Henderson et al. (1990), Mathies et al. (1991), Oesterhelt et al. (1992), Rothschild (1992), El-Sayed (1993), Ebrey (1993), Lanyi (1993), Balashov and Ebrey (1994)]. Asp85 is deprotonated in the purple membrane, but upon decreasing the pH to form the blue membrane (pK_a = 2.6 in 150 mM KCl) (Fischer, & Oesterhelt, 1979; Mowery et al., 1979; Jonas & Ebrey, 1991), it becomes protonated (Subramaniam et al., 1990; Metz et al., 1992). Studies of mutants of bR in which Arg82 was replaced with a neutral residue, alanine or glutamine, indicate that Arg82 strongly affects the pK_a of the purple-to-blue transition (pK_a of Asp85) (Stern & Khorana, 1989; Subramaniam et al., 1990; Balashov et al., 1993; Brown et al., 1993). In the R82A and R82Q mutants expressed in *Halobacterium salinarium,* the pK_a of Asp85 was shifted from 2.6 in the WT to 7.2−7.5 (Balashov

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[1] Abbreviations: bR, bacteriorhodopsin; WT, wild type; LA, light-adapted; DA, dark-adapted; k_{da}, rate constant of dark adaptation; f_{D85H}, fraction of protonated Asp85 (fraction of blue membrane).

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et al., 1993; Brown et al., 1993), which makes these mutants functionally inactive at pH <6.

Light-initiated photochemical changes lead to a decrease in the pK_a of the Schiff base and probably an increase in the pK_a of Asp85, which results in deprotonation of the Schiff base in the M intermediate (Govindjee et al., 1994). The proton goes to Asp85, but FTIR data indicate that Asp85 does not release its proton directly into the solution (Braiman et al., 1988; Müller et al., 1991; Rothschild, 1992; Souvignier & Gerwert, 1992); rather, an unknown group, X, must be the proton release group (Ebrey, 1993; Lanyi, 1993). The interaction between Asp85 and Arg82 plays an important role in proton release and in thermal isomerization (Balashov et al., 1993), since both processes are drastically affected in the R82A and R82Q mutants expressed in *Escherichia coli* (Dunach et al., 1990; Otto et al., 1990) and in *H. salinarium* (Balashov et al., 1993). These mutants show delays in the kinetics of proton release (Balashov et al., 1993; Brown et al., 1994a), which implies that Arg82 is associated with the proton release pathway of bR. An intriguing possibility is that Arg82 is the group that releases the proton (Balashov et al., 1993).

In all of the previous mutant studies the positively charged Arg was replaced by neutral residues (alanine and glutamine). In order to further characterize the roles of the positive charge and the guanidinium group of R82, we constructed the R82K mutant in which Arg82 was substituted with lysine. Like Arg, Lys is presumably positively charged in bR near neutral pH. Thus, one may expect that those features primarily associated with the positive charge in the 82 position would more closely resemble the corresponding features in the WT, rather than those in R82A(or Q) mutants. Lysine lacks a guanidinium group that may be important in hydrogen bonding to several other residues in the proton release channel. Features that primarily depend on the presence of the guanidinium group would be altered in the R82K mutant, as in the R82A mutant. In solution the pK_a of the ϵ-amino group of Lys is about 1.7 units less than the pK_a of the guanidinium group of Arg (ca. 12). If this difference is preserved for the Arg82 and Lys82 residues in the pigment, then one may expect that pH dependencies that are controlled by Arg in the WT will be shifted about 1.7 units to lower pH in the R82K mutant. In this context, we examined the effects of the Arg82 → Lys mutation in bR on basic spectral, photochemical, and functional properties of the R82K mutant. We were specifically interested in the following aspects.

(1) Role of Positive Charge at Position 82 in Determining the pK_a of Asp85. Since the electrostatic interaction between Asp85 and Arg82 is important in keeping the pK_a of Asp85 low (Stern & Khorana, 1989; Balashov et al., 1993), one may expect that the positive charge on Lys would lower the pK_a of Asp85 from the value of 7.2 observed in the R82A and R82Q mutants to a value close to that in the WT (2.6). If this is true, then removal of this positive charge on Lys82 due to deprotonation (at high pH) or by chemical modification should increase the pK_a of Asp85.

(2) Role of Arg82 in the Control of the pH Dependence of the Rate Constant of Dark Adaptation (Thermal Trans→13-Cis Isomerization). In the WT, the rate constant of dark adaptation, k_{da}, is controlled by two groups with pK_{a1} = 2.6 and pK_{a2} = 9.7 (in 150 mM KCl) (Ohno et al., 1977; Warshel & Ottolenghi, 1979; Balashov et al., 1993). This is drastically changed in the R82A mutant, in which the pH dependence of k_{da} has only one pK_a = 7.5, the same as the pK_a of Asp85 in this mutant (Balashov et al., 1993). In the R82A mutant the second group (which we call X′) is either missing (which means that X′ is Arg82) or has an altered pK_a, so that it does not noticeably contribute to the observed pH dependence of dark adaptation between pH 5.5 and 10. An interesting question in this context is how the R82→K mutation affects the two pK_a values of the pH dependence of dark adaptation. In particular, the R82K mutant should be useful in testing the hypothesis that the second residue (X′) is Arg82 or that the pK_a of X′ is controlled by Arg82. Brown et al. (1993) estimated that the pK_a of Arg82 in bR is high (13.8) and, therefore, considered it unlikely to act as the proton release group X (or the group X′ controlling thermal isomerization). On the other hand, a comparison of the pH dependence of dark adaptation in the WT and R82A implies that the pK_a of Arg82 may be low (pK_a ~ 9.5) (Balashov et al., 1993).

(3) Mechanism of Thermal Isomerization and Control of the pK_a's of Asp85 and X′ in bR. In R82A, the rate constant of dark adaptation is proportional to the fraction of protonated Asp85, which indicates that thermal isomerization of the chromophore proceeds through the transient protonation of Asp85 (Balashov et al., 1993). We have proposed that protonation of Asp85 catalyzes thermal isomerization not only in the R82A mutant but also in the WT and other mutants of bR. That implies proportionality between the rate constant of dark adaptation and the fraction of the blue membrane. So far this proportionality was established only for the R82A mutant (Balashov et al., 1993). Correlation between the increase in the rate of dark adaptation at low pH and the purple-to-blue transition was pointed out earlier by Ohno et al. (1977) and Warshel and Ottolenghi (1979). In order to explain the complex pH dependence of the rate constant of dark adaptation in the WT, we suggested that in the WT Asp85 also has a complex titration curve, which can be described by a model of two interacting residues, Asp85 and X′. According to this hypothesis, deprotonation of X′ with pK_a = 9.7 in the WT causes an increase in the pK_a of Asp85 from 2.6 to 7.5. Conversely, the protonation of Asp85 decreases the pK_a of X′ by about 4.9 units in the WT, and a similar (or slightly smaller) decrease in the pK_a of X′ is expected upon the formation of the M intermediate (Balashov et al., 1993). Thus, X′ can act as the proton release group X postulated by Zimányi et al. (1992). The hypotheses that the pK_a of Asp85 is controlled by X′ and that protonation of Asp85 is the key factor in the chromophore thermal isomerization have two experimentally testable predictions: (i) the titration curve of Asp85 (blue membrane) should deviate from the simple case for a single residue (Henderson−Hasselbalch equation) and follow a more complex pH dependence; (ii) the rate constant of dark adaptation should be directly proportional to the fraction of protonated Asp85. In the R82K mutant at neutral and high pH, the fraction of the blue membrane is larger than in the WT and can be more easily detected. This enabled us to test the predictions of the model.

(4) Involvement of Arg82 in Light-Induced Proton Release. The R82A and R82Q mutants exhibit drastic delays in proton release, indicating that Arg82 is involved in the catalysis of proton release (Otto et al., 1990; Balashov et al., 1993). The question is whether lysine can effectively substitute for Arg82 and correct the defect in proton release produced by the R82A and R82Q mutations. A more specific question is whether Arg82 is itself the proton release group or whether

it merely controls the pK_a of another residue (X) acting as a proton release group (or the pK_a of a group of interacting residues forming a proton release complex; see discussion in Balashov et al. (1993); Brown et al. 1993; Lanyi, 1993).

(5) Factors Controlling the Rate of the Schiff Base Deprotonation and Formation of the M Intermediate. Is the rise time of the M intermediate fast (1 μs) and pH-independent in R82K, as in R82A, or slow (100 μs at pH 7) and pH-dependent, as in the WT? Which of the photocycle reactions (L→M→N→O→bR) are affected by the R82K mutation? We also wanted to determine whether alterations in the 13-cis photocycle, noticed in R82A, are also present in the R82K mutant. In this paper, we characterize basic spectral, kinetic, and photochemical properties of the R82K mutant of bR and try to address these questions.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. E. coli BMH71−18 *mutL* (Kramer et al., 1984) was used for site-directed mutagenesis. *E. coli* JM101 was used to propagate mutant phages, and competent *E. coli* NovaBlue (Novagen, Madison, WI) was used for transformation of the pT7 Blue vector (Novagen, Madison, WI) containing PCR-generated fragments. All *E. coli* strains were grown in 2× YT or LB medium (Ausubel et al., 1989). The wild-type and R82K *bop* genes were expressed in *H. salinarium* IV-8 (a gift of Richard Needleman), which contains a stable IS*H1* insert in the *bop* gene (DasSarma et al., 1984). *H. salinarium* IV-8 was grown on C medium (Needleman et al., 1991).

Site-Directed Mutagenesis of bR. Arg82 was replaced with Lys by oligonucleotide-directed, site-specific mutagenesis of the 2.7 kb *Bam*HI−*Hin*dIII *bop* gene insert ligated in M13mp19 using the mutagenic primer 5′ CTGG GCG AAG TAC GCT GAC3′, as described by Menick (1991). Mutants were initially screened by colony blot hybridization, and the entire coding region of the *bop* gene was sequenced to ensure that no additional mutations occurred. The *Bam*HI−*Hin*dIII *bop* gene insert was restricted from the M13mp19RF and religated into pMC-1.

Transformation of H. salinarium IV-8. The R82K bR mutant and wild-type control were transformed into *H. salinarium* strain IV-8, which contains the IS*H1* insert within the *bop* gene. Transformation was performed as described (Balashov et al., 1993). Several independent clones were picked and struck out on growth medium plates; single colony isolates were grown in 2 L of growth medium (Needleman et al., 1991) to characterize each bR mutant. Southern blot analysis was carried out as described (Ausubel et al., 1989). Genomic DNAs from *H. salinarium* IV-8 cells and IV-8 cells transformed with wild-type or the R82K mutant *bop* genes were digested with *Pst*I, transferred to Gene Screen Plus hybridization transfer membranes (New England Nuclear Research Products, Boston, MA), and hybridized with a ^{32}P-labeled 500 bp *Kpn*I *bop* gene fragment. In addition, the R82K *bop* gene was amplified for sequencing by polymerase chain reaction (PCR). PCR was performed with sheared *H. salinarium* genomic DNA as template, using the conditions described previously (Balashov et al., 1993). The 1150 bp PCR product was gel-purified and sequenced using the dsDNA cycle sequencing system (BRL, Grand Island, NY).

Absorption spectra and kinetics of dark adaptation were measured on a Cary-Aviv Model 14 spectrophotometer. Protonation of Asp85 was monitored through formation of the blue membrane, which was measured spectroscopically. All measurements were performed at 20 °C. *Flash-induced absorbance changes during the photocycle*, and measurements of light-induced transient proton release and uptake using the pH-sensitive dye pyranine were carried out as described previously (Balashov et al., 1993). *Spectral and kinetic data processing* was performed using a VAX Station 3100 and PV-Wave utilities, as described by Nelson (1991). Preparations of phosphatidylcholine vesicles containing R82K were similar to those described for the WT (Govindjee et al., 1980).

Chemical modification with acetic anhydride was performed similarly to Takeuchi et al. (1981) and Maeda et al. (1982) with some variation of conditions. A 1 mL suspension of light-adapted R82K (OD 2.2 units) was mixed with 1 mL of saturated sodium acetate (final concentration 2.5 M) and cooled to 1 °C. Four microliters of acetic anhydride was added every 10 min. The total time of incubation was 80 min. Addition of acetic anhydride and its subsequent hydrolysis caused a decrease in pH, which was compensated by the addition of 3−5 μL of 10 M NaOH. Buffer (120 mM Mops, pH 8.0) was used in order to maintain pH during modification within a narrower, ±0.1 pH unit, range. NaOH (1 M) was used to adjust the pH in the presence of buffer. In order to achieve a larger degree of modification, the amount of acetic anhydride was increased to 5 μL aliquots, which were added every 5 min to a 1.5 mL suspension of membranes (R82K and the WT) in 2.5 M sodium acetate. The time of incubation was 120 min. After modification, the sample was diluted with 30 mL of ice cold 10 mM Ches buffer (pH 9.2) and incubated for 1 h in the dark. The sample was subsequently washed twice, resuspended in 0.7 mL of 150 mM KCl (or 75 mM K_2SO_4), and used for absorption measurements and titration.

RESULTS

pH Dependence of the Absorption Spectrum of R82K: Purple-to-Blue Transition at Low pH. Formation of the blue membrane of R82K at low pH is accompanied by the absorption changes shown in Figure 1. Upon a decrease in the pH from 6.5 to 2.2 (in 75 mM K_2SO_4), the maximum in the absorption spectrum of dark-adapted R82K shifts by 59 nm, from 552 to 611 nm (Figure 1A). The absorption maximum of the blue membrane (611 nm) is closer to that in the WT (603 nm) and significantly (15 nm) shifted to the red compared to that in R82A (596 nm). The maxima in the difference spectra pH_i minus pH 6.5 are at 630 nm, minima are around 532−535 nm, and the isosbestic points are at 580 nm in the pH range 6.5−3.3 and at 575 nm at pH below 3.3 (Figure 1B). The pK_a of the purple-to-blue transition in 75 mM K_2SO_4 is 3.44 ± 0.03, as determined from the absorption changes at 630 nm (Figure 2). Similar values for the pK_a were obtained from a fit of the absorption changes at 680 nm (where the contribution from the transition causing a slight shift in the isosbestic point at pH <3.3 is negligible): in the dark-adapted suspensions the pK_a was 3.52 ± 0.03 in 75 mM K_2SO_4; in gels, pK_a = 3.58 ± 0.05; in 150 mM KCl the pK_a was 3.61 ± 0.04. Thus, the pK_a of the purple-to-blue transition in the dark-adapted R82K is 3.6 in 150 mM salt.

The pK_a in R82K is closer to that in the WT (2.6) than to the corresponding pK_a in R82A (7.2). The fit of the titration

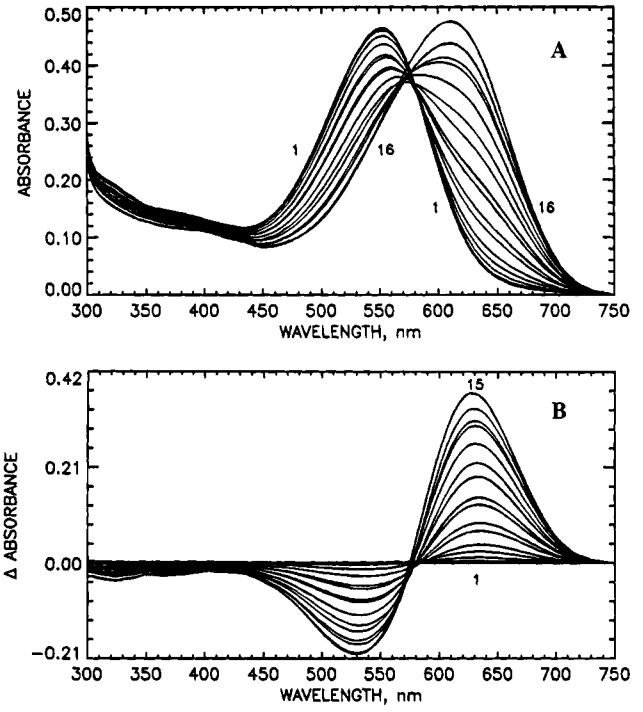


FIGURE 1: Purple-to-blue transition in R82K. (A) Spectra 1−16: absorption spectra of a dark-adapted suspension of R82K measured at pH 6.50, 5.64, 5.14, 4.75, 4.46, 4.08, 3.96, 3.75, 3.54, 3.44, 3.29, 3.13, 2.94, 2.87, 2.64, and 2.19 in the presence of 12 mM citric acid and 150 mM K^+ (75 mM K_2SO_4). (B) Spectra 1−15: difference spectra pH_i (listed in 1) minus pH 6.50.

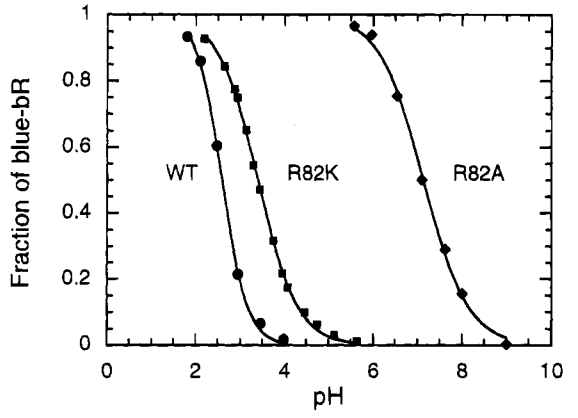


FIGURE 2: pK plots of the purple-to-blue transition in R82K, R82A, and WT membranes. The fraction of blue membrane in R82K was determined from absorbance change at 630 nm (see Figure 1B). Data for WT were obtained in a similar experiment. Data for R82A are taken from Balashov et al. (1993), where the absorbance changes were measured at 618 nm. The data were fit with equation $f = 1/(1 + 10^{n(pH-pK_a)})$; pK_a = 2.6, 3.4, and 7.2 for the WT, R82K, and R82A, respectively, and n = 1.0 for the R82K and R82A and 1.5 for the WT.

curve shows that one H^+ is involved in the purple-to-blue transition in R82K (n = 1 in the equation describing the titration curve, see Figure 2). A similar coefficient was found in R82A (Balashov et al., 1993), while in the WT, in agreement with earlier observations (Mowery et al., 1979; Jonas & Ebrey, 1991), a better fit is obtained by assuming n = 1.5.

Second Acid-Induced Transition at Low pH. At pH below 2, R82K undergoes another transformation that is accompanied by a 21 nm blue shift of the absorption spectrum (from 611 nm at pH 2.2 to 590 nm at pH 1.0) and a decrease in extinction (Figure 3A). The minimum in the difference spectrum of this transition (pH 1.0 minus pH 2.2) is at 632

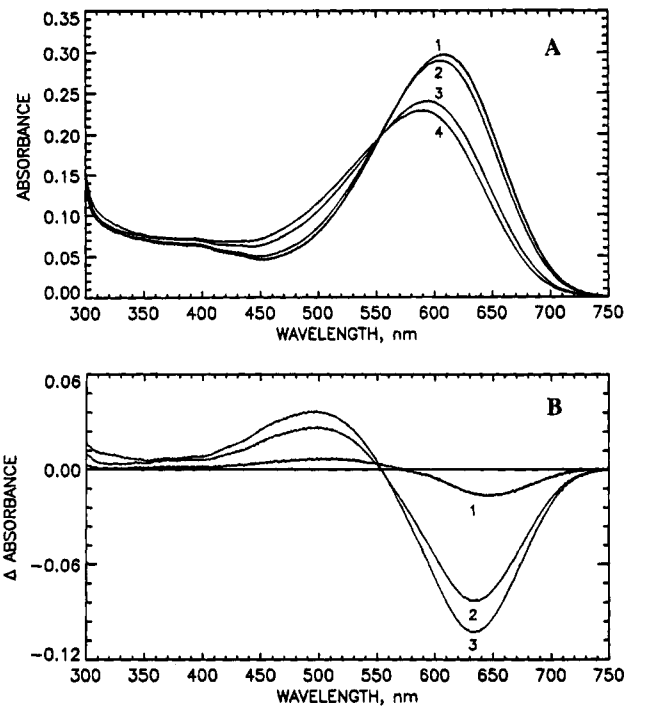


FIGURE 3: (A) Absorption spectra of dark-adapted R82K gels in 75 mM K_2SO_4 taken at different pH's: 1, pH 2.2; 2, pH 1.8; 3, pH 1.4; 4, pH 1.0. (B) spectra 1−3: difference absorption changes produced by a decrease in pH from 2.2 to 1.8 (1), 1.4 (2), and 1.0 (3). Gels were incubated at the given pH for 12 h in the dark at room temperature.

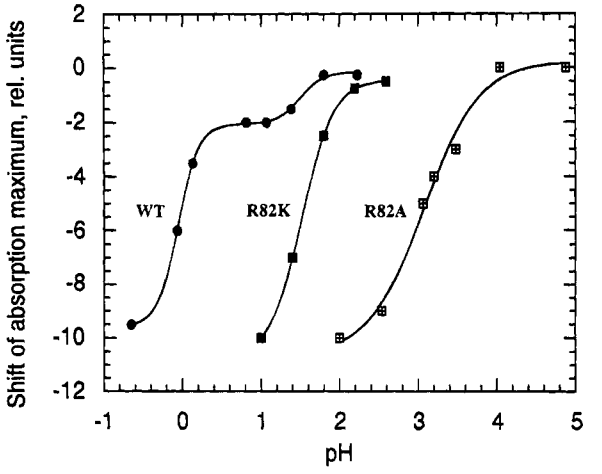


FIGURE 4: pK plots of the absorption shift to shorter wavelengths in the WT, R82K, and R82A at low pH in 20 mM K_2SO_4. Upon decreasing the pH, the absorption maximum of the blue membrane in the pigments shifts to shorter wavelength (from 603 to 584 nm in the WT, from 585 to 574 nm in R82A, and from 611 to 590 nm, in R82K). On the y axis the arbitrary units are plotted in order to scale the shifts in the pigments. To get the absolute value of the blue shift (in nm) at a given pH, the relative units should be multiplied by 2 for the WT and R82K and by 1 for R82A.

nm, the isosbestic point is at 555 nm, and the maximum is at 500 nm (Figure 3B). The pK_a of this transition is 1.5, n = 3 in 20 mM K_2SO_4 (Figure 4). The large value of n suggests that the protonation of several groups, possibly phospholipids and perhaps also Asp212, takes place. A similar transition is also observed in R82A and in the WT (Balashov et al., 1993). In R82A it is accompanied by a smaller, 13 nm blue shift of the absorption band with pK_a = 3.0 in 1 M Na_2SO_4. In 20 mM K_2SO_4 the pK_a of this transition is 3.1, so that it does not depend much on salt concentration.

In the WT, in 20 mM K_2SO_4 a 19 nm blue shift takes place (from 603 to 584 nm) when the pH is decreased from 2 to −1. The pK_a of the main transition is 0, which is 3 pH units less than that in R82A and 1.5 units less than that in R82K (Figure 4). Thus, the pK_a of this second transition, like the purple-to-blue transition, is sensitive to substitutions of Arg82 and apparently is caused by the protonation of a residue close to Arg82 (Asp212 is the most probable candidate). Since chloride ions were absent, this transition is not caused by the formation of the acid purple species, which requires Cl^- (Fischer & Oesterhelt, 1979; Mowery et al., 1979).

Red Shift of the Chromophore and Tryptophan Absorption Bands at High pH. The absorption maximum of the dark-adapted R82K undergoes a small red shift (from 552 to 554 nm) upon changing the pH from 6.5 to 9.2, with a pK_a around 7.6 (Figures 5A and 6A). The difference spectrum (Figure 5B) shows peaks at 590, 295, and 286 nm. The latter two are due to a small red shift of a Trp absorption band (Balashov et al., 1988, 1991). The shifts of both the chromophore and Trp absorption bands are completely reversible. The amplitude of absorption changes at 590 nm, produced by change in the pH from 9.2 to 6.5, is larger in the light-adapted (at pH 9.2) R82K than in the dark-adapted R82K. This indicates that mostly the chromophore absorption band of *trans*-bR undergoes a red shift. The pH-induced shift of Trp bands is the same in light-adapted and dark-adapted membranes. The pK_a of the Trp absorption shift in R82K is 7.6, while in the WT it is ca. 9.0. Thus, the shifts of the chromophore and Trp absorption bands are sensitive to the Arg82→Lys substitution.

Blue ↔ Purple Transitions in R82K at High pH. The Second pK_a *in the Titration Curve of Asp85.* We have recently suggested (Balashov et al., 1993) that, besides its major transition at pK_a ~ 3, Asp85 may have a complex titration curve with a second pK_a transition at high pH due to the interaction of Asp85 with a second group X′. The R82K mutant appears to be a good system to test this hypothesis since it has a larger amount of blue membrane at neutral pH than the WT. The blue-to-purple transition in R82K can be easily detected at pH >6. Figure 5C shows a portion of the difference spectra obtained in a suspension of dark-adapted R82K membranes upon increasing the pH from 6.5 to 9.2. The negative bands seen in the spectra pH_i minus pH 6.5 at 650−700 nm reflect the decrease in the amount of blue membrane upon increasing the pH. These changes are completely reversible. At wavelengths shorter than 650 nm, an absorption increase is observed due to the red shift of the chromophore absorption band (Figure 5B). At wavelengths longer than 670 nm, the contribution from this shift is negligibly small and the absorbance changes are caused by the transformation of the blue membrane into purple. The absorption changes at 680−700 nm are proportional to the changes in the fraction of blue membrane. The plot of the absorption changes at 680 nm versus pH shows the blue-to-purple transition with a pK_a = 7.8−8.0 (Figures 6B and 9B).

Light Adaptation: Photoreversibility and pH Dependence. Light adaptation of R82K produces absorption changes similar to those observed in the WT; however, in contrast to the WT, the amplitude of absorbance changes and the absorption maximum of the light-adapted suspension of R82K depend on pH (Figures 6A and 7A) and, below pH 9, on the wavelength of illumination (Figure 7B). At pH 5.6

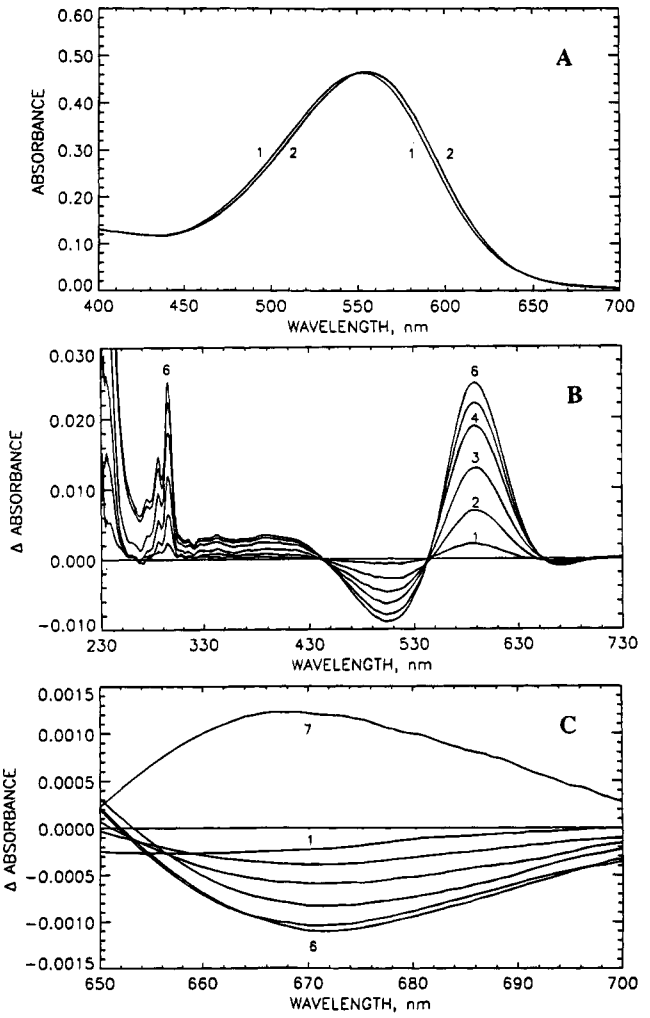


FIGURE 5: Absorption changes in a dark-adapted suspension of R82K in 150 mM KCl upon increasing the pH from 6.5 to 9.2. (A) Red shift of the chromophore absorption band of R82K at alkaline pH: 1, pH 6.5; 2, pH 9.2. (B) Curves 1−6: difference absorption spectra pH_i minus pH 6.5 produced by increases in pH in the dark from pH 6.5 to pH 6.8, 7.3, 7.7, 8.1, 8.6, and 9.2. (C) *Curves 1−6*: a portion of the difference spectra pH_i minus pH 6.5 shown in panel B. The negative band at 650−700 nm is due to the blue-to-purple transition in R82K. At wavelengths shorter than 670 nm, this negative band overlaps with absorption changes of opposite sign due to a 2 nm red shift of the chromophore absorption band. *Curve 7*: absorption changes produced by a decrease in pH from 9.2 to 6.4 in order to demonstrate the reversibility of absorbance changes at 650−700 nm at high pH. The absorbance of the sample at the maximum (552 nm) was 0.46. To maintain the pH between 6 and 10, a mixture of several buffers (Mes, Mops, Tricine, Ches, and Caps), each at 0.5 mM, was used. To change the pH, each time 1 μL of 1/6 N KOH was added to a 700 μL suspension of R82K membranes.

the maximum in the light-adapted spectrum is at 558 nm, while at pH 9.2 it is at 565 nm. The pK_a of the shift of the absorption maximum of light-adapted R82K is around 7.7 (Figure 6A). The pH dependence of the absorption maximum of light-adapted R82K is partially due to the photoreversibility of light adaptation below pH 9. Illumination at 610 nm partially reverses absorption changes produced by blue light illumination and causes a decrease in the amount of *trans*-bR in the light-adapted sample (Figure 7B). The latter phenomenon indicates that, between pH 5.6 and 9.2, light causes the transformation not only of the 13-*cis* into the *trans* form of the pigment but also the reverse *trans*→13-*cis* photoreaction, which prevents complete conversion of 13-*cis*- into *trans*-bR at these pH's. At 520 nm the

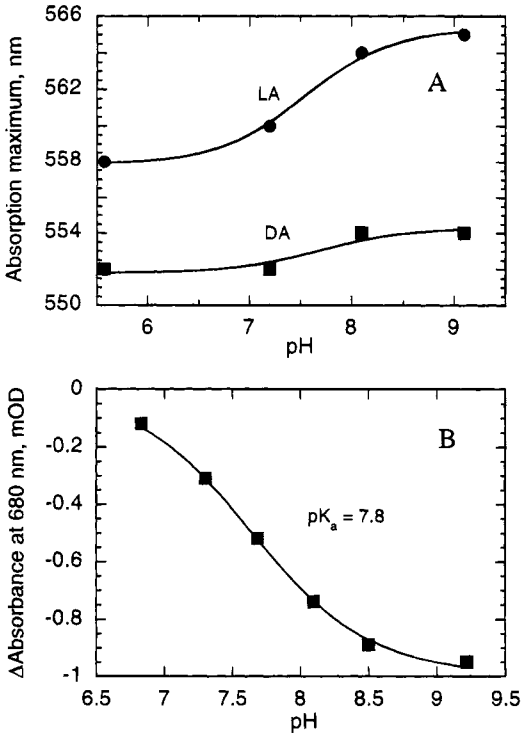


FIGURE 6: (A) pH dependence of the absorption maximum of dark-adapted and light-adapted R82K in 150 mM KCl. (B) pH dependence of absorption changes at 680 nm due to the blue-to-purple transition in the dark-adapted suspension of R82K. Data were taken from the difference spectra pH_i minus pH 6.5 shown in Figure 5C and fitted with the Henderson–Hasselbalch equation: pK_a = 7.8, ΔA_{680} = 1.0 mOD.

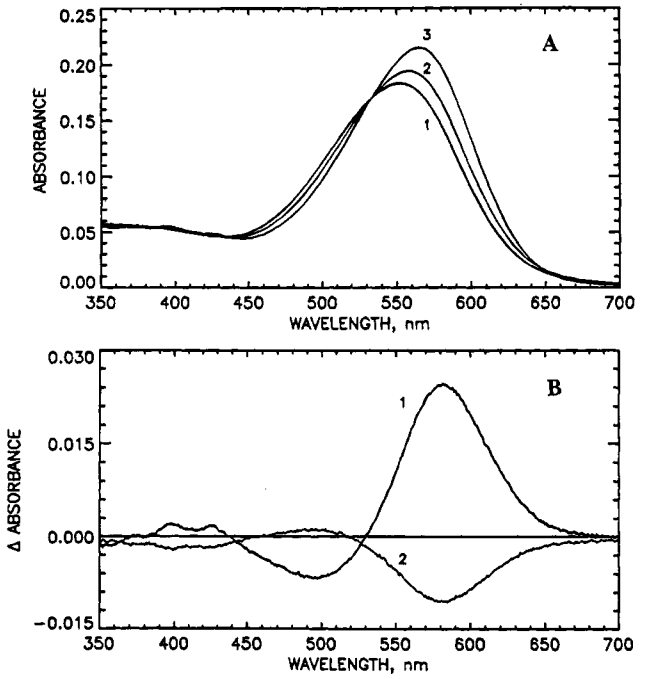


FIGURE 7: pH dependence of light adaptation of R82K in 150 mM KCl and its photoreversibility. (A) Absorption spectra: 1, dark-adapted suspension of R82K at pH 5.6; 2 and 3, light-adapted with 520 nm light at pH 5.6 and pH 9.2, respectively. (B) Photoreversibility of light adaptation at pH 7.6. Absorption changes produced by 1, light adaptation at 520 nm (LA at 520 nm minus DA); 2, additional 2 min of illumination at 610 nm (LA at 610 nm minus LA at 520 nm).

extinction of the 13-*cis* pigment is larger than that of the trans pigment, and illumination at this wavelength converts the maximal amount of 13-cis into trans. At pH >9, the

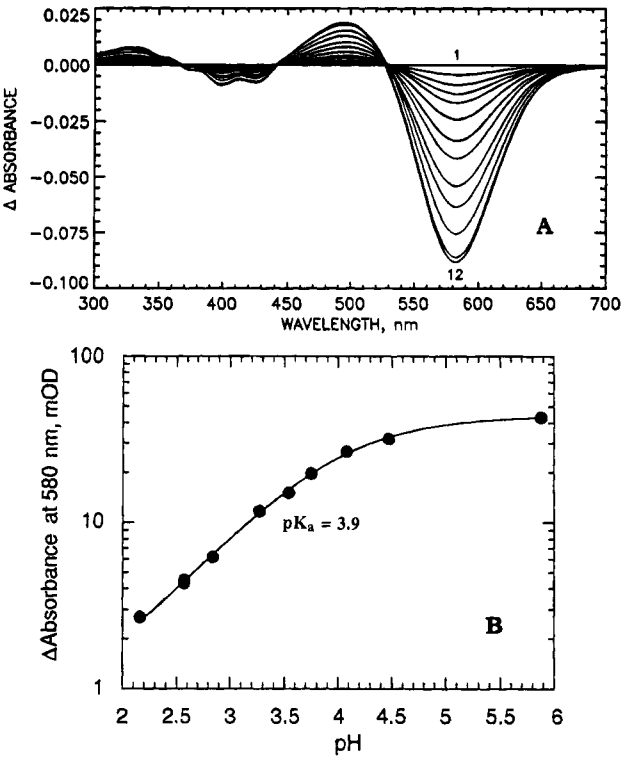


FIGURE 8: (A) Absorption changes during dark adaptation of R82K at pH 7.6 in 150 mM KCl. The difference spectra DA minus LA (1–12) were taken 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 270, and 300 min after light adaptation. (B) pH dependence of absorption changes at 580 nm associated with light–dark adaptation. Solid line is the fit with the Henderson–Hasselbalch equation: pK_a = 3.9, n = 1.

photochemical transformation of *trans* into 13-*cis* is inhibited, and light-adapted samples do not contain 13-*cis*-bR (see the following). By measuring the time to light-adapt the R82K versus WT membranes under continuous illumination at 510 nm, we estimated that the quantum efficiencies of light adaptation in R82K and the WT are approximately equal (at pH 9).

Dark Adaptation. Upon incubation in the dark, the light-adapted R82K membranes undergo absorption changes that are opposite to those produced by light adaptation (Figure 8A). These changes presumably are due to the same process observed in the WT: the thermal isomerization of the chromophore from *all-trans*,15-*anti* into the 13-*cis*, 15-*syn* configuration (Oesterhelt et al., 1973; Harbison et al., 1984). The pK_a of the ϵ-amino group of free Lys is about 1.7 units less than that of the guanidinium group of Arg. If Arg is indeed the second group controlling the rate of dark adaptation in bR with pK_{a2}, then one may expect that, upon substitution of Arg by Lys, pK_{a2} will shift to lower pH by about 1.7 units.

The rate constant of dark adaptation, k_{da}, in R82K was obtained from the time course of the absorption changes at 580 nm in the dark immediately after light adaptation. The kinetics at all pH's were monoexponential. From the fit of the kinetic curves with the simple equation $\Delta A_{580}(t) = \Delta A_{580} \exp(-k_{da}t)$, the values for k_{da} were determined with an accuracy of ±5–10%. The pH dependence of k_{da} in R82K is shown in Figure 9A. The maximum value of the rate constant, k_{da}^0 = 0.021 s^{-1}, is observed at pH 2.2, at which more than 90% of Asp85 is protonated (as follows from the pK_a plot of the purple-to-blue transition in Figure 2). This value is 2 times smaller than that observed in the WT (0.04

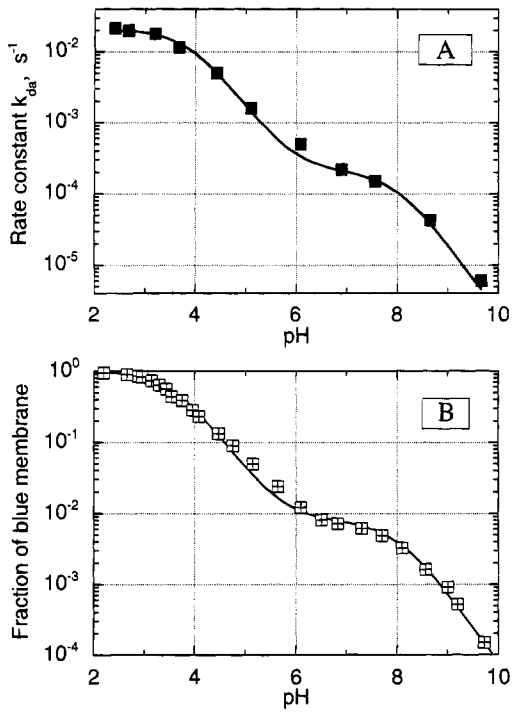


FIGURE 9: (A) pH dependence of the rate constant of dark adaptation, k_{da}, in R82K. The fit was obtained by using eqs **1** and **2** with the following parameters: $k_{da}° = 0.021$, $pK_{a1} = 3.9$, $pK_{a2} = 8.0$, and $pK_{a3} = 5.9$ (see text for details). (B) Fraction of pigment having protonated Asp85 (blue membrane) in R82K calculated from the absorption changes at 680 nm, which are shown in Figure 1B and Figure 5C. The data were fit using eq **2**. The fit is given for $pK_{a1} = 3.7$, $pK_{a2} = 8.0$, and $pK_{a3} = 5.7$.

s^{-1}) and 5 times larger than that in R82A (0.0045 s^{-1}) (Balashov et al., 1993). As with the WT, the pH dependence of k_{da} indicates that two amino acid residues control thermal isomerization in R82K, with $pK_{a1} = 3.9$ and $pK_{a2} = 8.0$. The first one is close to the pK_a of the main purple-to-blue transition ($pK_a = 3.6$) and is associated with the change in protonation of Asp85. The $pK_{a2} = 8.0$ reflects the deprotonation of X′ (see the following).

The data shown in Figure 9A were fitted by the model (Balashov et al., 1993), which proposes that the rate constant k_{da} is proportional to the fraction of protonated Asp85 (fraction of blue membrane; see eq **1**). The latter is determined by an equation taking into account the interaction of Asp85 and a residue X′, which affects the pK_a of Asp85 (see eq **2** in discussion). The fit uses parameters, $pK_{a1} = 3.9$, $pK_{a2} = 8.0$, and $k_{da}° = 0.021$ s^{-1}, as well as a new fourth parameter, the pK_a of group X′ when Asp85 is protonated (pK_{a3}). This appears to be 5.7. It also follows from the fit of the data that the pK_a of Asp85 at high pH (when X′ is deprotonated) would be 6.0. Evidence in favor of this model was obtained upon direct determination of the pH dependence of the fraction of protonated Asp85, f_{D85H}, measured as the fraction of blue membrane over a wide range of pH values (see Figure 9B), and comparison with the pH dependence of k_{da}.

Proportionality between the Rate Constant of Dark Adaptation and the Fraction of Protonated Asp85. Recently we suggested that transient protonation of Asp85 is the key factor controlling the rate of thermal isomerization in bR (Balashov et al., 1993). This hypothesis predicts that the rate constant of dark adaptation, k_{da}(pH), should be directly proportional to the fraction of protonated Asp85, f_{D85H}(pH):

$$k_{da}(\text{pH}) = k_{da}° f_{D85H}(\text{pH}) \quad (1)$$

where $k_{da}°$ is the constant that is equal to the rate of isomerization in the state of blue membrane. The data we obtained on the R82K mutant support this hypothesis. Figure 9B depicts the fraction of the blue membrane (estimated from the absorption changes at 680 nm shown in Figures 1B and 5C) versus pH on a log scale. In addition to the main transition with $pK_a = 3.7$, a second transition with $pK_a \sim 8.0$ is present. The key point is that the pH dependence of the fraction of blue membrane is very close to the pH dependence of the rate constant of thermal isomerization (Figure 9A,B). The pK_a values in the fits of k_{da} and f_{D85H} are identical, except for a small difference in the value for pK_{a1} (3.9 in the fit of k_{da} and 3.7 in the fit of f_{D85H}). This difference may originate from the fact that titration of the purple-to-blue transition was made in the dark-adapted state, while the rate constants of dark adaptation were determined by starting from the light-adapted state. In dark-adapted wild-type bR, the pK_a of the purple-to-blue transition is slightly (about 0.3 pH unit) shifted to lower pH (Mowery et al., 1979), indicating that the pK_a of Asp85 may be slightly lower in 13-*cis*-bR than in *trans*-bR (Balashov et al., 1995). This is supported by the fact that the pH dependence of absorption changes at 580 nm due to dark adaptation (which are proportional to the amount of *trans*-bR purple) has a higher pK_a (3.9, Figure 8B) than was observed in the titration of dark-adapted membranes (3.5−3.6). Taking into account these minor differences, we conclude that eq **1** is a quite accurate description of the pH dependence of thermal isomerization. The ratio of the rate constant k_{da}(pH) and the fraction of protonated Asp85, f_{D85H}(pH), is indeed almost constant (within a factor of 2) over the pH range between 2 and 9. This proves the proportionality between the rate of thermal isomerization and the fraction of protonated Asp85.

Alterations in the Photochemical Cycle of R82K. The yield of the M intermediate in R82K is pH-dependent. Light-induced absorbance changes at 412 nm, ΔA_{412}, are 2 times larger at pH 9.2 than at pH 5.6 (Figure 10). The pK_a of the increase in ΔA_{412} is 8.0. The pH dependence of M yield apparently reflects the pH dependence of the fraction of *trans*-bR in the light-adapted membranes and can be explained by the photoreversibility of light adaptation, along with the inability of 13-*cis*-bR to form M.

The rate of M formation is very fast in R82K (Figure 11). About 80% of M is formed with a time constant 1.5 μs. A second component with $\tau \approx 8$ μs is also present. The first time constant ($\tau \approx 1.5$ μs) does not show any significant pH dependence.

The rate of M decay is fast at pH 5.6 (about 3 ms) and is faster than pigment recovery at this pH (see the 410 and 570 nm traces in Figure 12A). The O intermediate does not accumulate (Figure 12A, trace at 630 nm), suggesting that the rate-limiting step in the photocycle is the decay of the N intermediate (thermal isomerization from 13-*cis* into *trans* configuration). At high pH, M decay exhibits a long-lived component that is apparently associated with the relaxation of the N intermediate (Figure 12B).

Photoproducts of 13-Cis Cycle. The presence of the 13-*cis* isomeric form of the pigment can be detected by studying, on the submillisecond time scale, the light-induced changes at 630−660 nm due to formation and decay of the batho-products of the 13-*cis* form (Sperling et al., 1977; Balashov et al., 1993). At pH 5.6, a large light-induced increase in

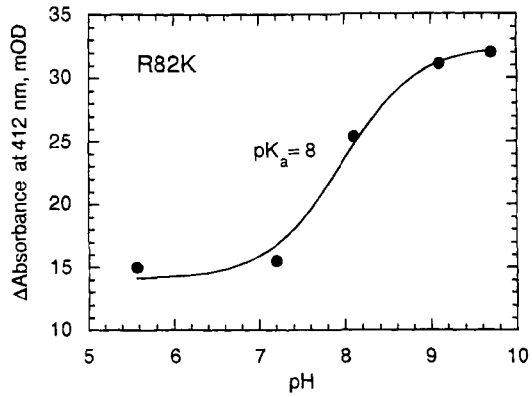


FIGURE 10: pH dependence of flash-induced absorption changes at 412 nm due to M formation in the suspension of R82K mutant in 150 mM KCl.

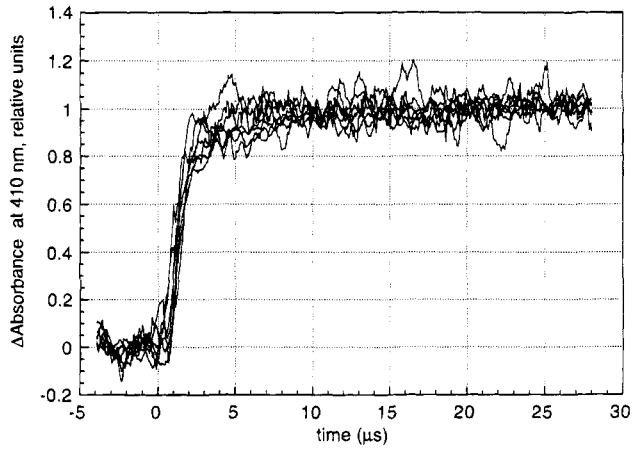


FIGURE 11: Kinetics of M formation (measured as flash-induced absorbance changes at 410 nm) in R82K in 100 mM KCl. Traces were taken at pH 3.6, 4.5, 5.4, 6.4, 7.1, 8.9, and 10.8 and normalized at the maximum absorbance.

absorption at 660 and 630 nm is observed in the light-adapted (and dark-adapted) samples (Figure 12C). These signals are absent at pH 9.2 and 10.3 from the light-adapted samples, but are present in the dark-adapted ones. They are associated with the formation of the photoproducts of the 13-*cis* pigment. The transient signal at 630 nm has several phases: a fast, unresolved rise is followed by a decrease in absorbance (due to the L to M transition in the *trans* cycle) and a subsequent 20 μs (approximately) rise followed by a 0.2 ms decay. At 660 nm the initial rise is relatively small and the decrease is absent; the 20 μs and 0.2 ms phases dominate the signal. Thus, similar to the R82A mutant, in R82K the bathoproduct from the 13-*cis* pigment decays much faster than in the WT (0.2 versus 30 ms). As in R82A (Balashov et al., 1993), it includes an unresolved initial phase and a subsequent increase in and decay of absorbance.

Light-Induced pH Changes in the Vesicles of R82K. Under continuous illumination of phosphatidylcholine vesicles containing R82K, the light-induced uptake of protons is observed (data not shown). The amplitude of the signal is about 40% of that in vesicles containing WT with the same concentration of the pigment and after correction for the different buffering capacities of the vesicles. These data indicate that R82K pumps protons with an efficiency ≥40% of that of the WT.

Light-Induced Proton Release and Uptake in Suspensions of R82K. Figure 13 shows the light-induced absorbance changes of the pH-sensitive dye pyranine in a suspension of

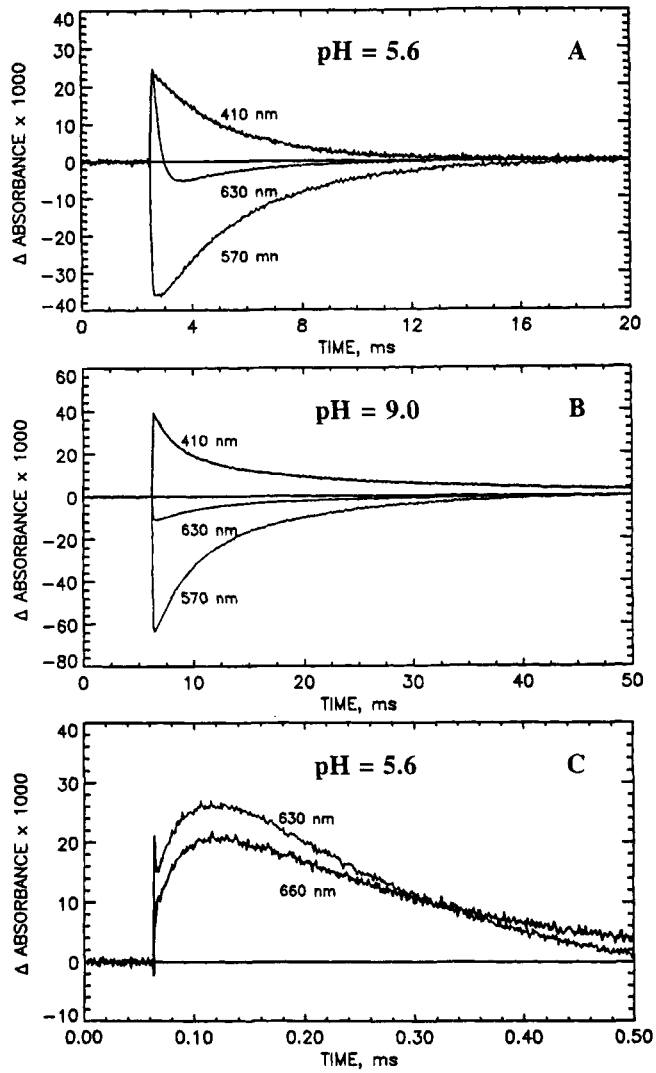


FIGURE 12: Flash-induced absorption changes at 410, 570, 630, and 660 nm in light-adapted R82K in 1 mM KCl: (A) pH 5.6; (B) pH 9.0. (C) Absorbance changes at 630 and 660 nm (due to photoproduct(s) of 13-cis pigment, see text) at pH 5.6. Excitation was with a laser pulse at 532 nm.

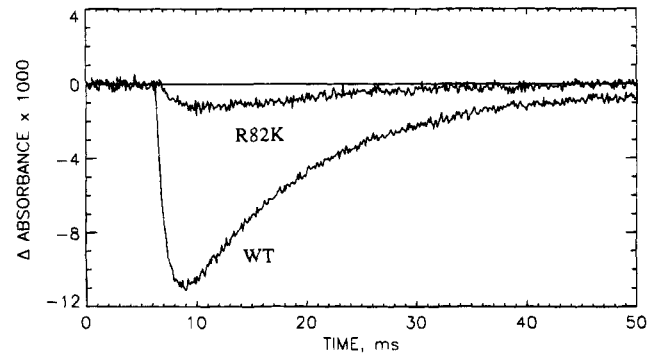


FIGURE 13: Flash-induced absorption changes at 460 nm of the pH-sensitive dye pyranine in suspensions of R82K and WT membranes at pH 7 in 1 M KCl. The amplitudes of absorbance changes at 412 nm were 32 mOD in R82K and in the WT.

R82K membranes and in the WT at pH 7. The kinetics of the signal is similar to that in the WT: fast release (τ = 1 ms) followed by the uptake (τ = 13 ± 2 ms) of protons. However, the amplitude of the pyranine signal is approximately 8 times smaller in R82K than in the WT under similar conditions. (The ratio of the absorbance changes of pyranine at 460 nm to the absorbance changes at 412 nm

due to M formation is 1/3 in the WT and about 1/20 in R82K.) This suggests that significantly fewer protons (about 15%) are released to the bulk solution within 1 ms in R82K compared to the WT. The pyranine signal in R82K is different from that in R82A (Balashov et al., 1993), where the release is very slow (rise time of about 30 ms) and occurs after the uptake.

Modification of R82K with Acetic Anhydride. Chemical modification of Lys82 in R82K was used as a test for its accessibility from the bulk solution and as a way to model the effect of deprotonation of Lys82 on the pK_a of Asp85. If this residue can be made neutral by modification, then the modified pigment can be used to determine the pK_a of Asp85 when the positive charge is removed from Lys82. We used acetic anhydride, which reacts with the amino groups of lysines and the phenolic groups of tyrosines in proteins. The product of the latter reaction is unstable and the reaction is reversible. Incubation at mild alkaline conditions is sufficient to deacetylate tyrosyl residues, but not the amino groups of lysines (Means & Feeney, 1971). Acetylation of the amino group of Lys converts it into an electrically neutral residue. If Lys82 is accessible for modification in the R82K pigment, then in the acetylated pigment the pK_a for Asp85 should be ≈6, as follows from the fit of the pH dependence of the blue membrane in R82K (Figure 9B), or 7, assuming that it should be similar to the pK_a of Asp85 in the R82A and R82Q mutants.

Initially, R82K was modified under conditions similar to those used by Maeda et al. (1982) for the WT. After modification, 25% of the R82K pigment was in the blue form at pH 6, which indicated that Lys82 was partially modified with acetic anhydride. In order to increase the fraction of modified R82K, we increased the time of incubation with acetic anhydride to 2 h with a buffer to maintain pH during modification (120 mM Mops at pH 8.0). The experiments were done in parallel on the WT and R82K. In the WT, prolonged incubation with acetic anhydride (2 h) causes a moderate (1.8 pH units) shift of the pK_a of Asp85, as follows from the increase in the pK_a of the purple-to-blue transition from 2.6 to 4.4. This effect was described by Maeda et al. (1982). The 1.8 pH unit shift in the pK_a of Asp85 was attributed to the acetylation of a lysine residue (Maeda et al., 1982).

The pK plot of absorbance changes due to the formation of blue membrane in R82K treated with acetic anhydride (monitored at 660 nm) shows that the blue membrane is formed with two pK_a's, 4.4 and 6.5 (Figure 14). The first pK_a (4.4) belongs to the fraction of pigment in which Lys82 was not modified. The second pK_a is apparently due to the purple-to-blue transition in the molecules in which Lys82 was acetylated. The fraction of these molecules estimated from the absorption changes at 660 nm is 52%.

DISCUSSION

Purple-to-Blue Transition at Low pH. The pK_a of the purple-to-blue transition in R82K (3.6 in 150 mM KCl) is much closer to its value in the WT (2.6) than in R82A (7.2) (Figure 2). This is in agreement with the hypothesis that the electrostatic interaction of Asp85 with Arg82 (Lys82 in R82K) is an important factor determining the pK_a of Asp85 (Stern & Khorana, 1989; Balashov et al., 1993). The shift in the pK_a of Asp85 in R82K compared to its value in R82A is 3.6 pH units (7.2 − 3.6 = 3.6), which is 80% of the shift

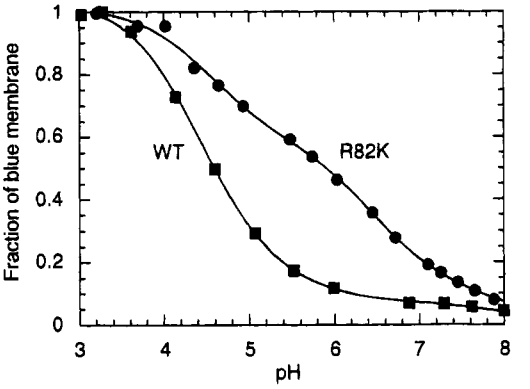
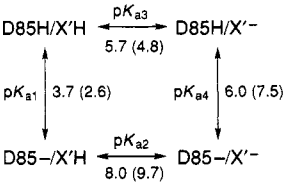


FIGURE 14: Comparison of the purple-to-blue transition in the WT and R82K (in 150 mM KCl) after incubation with acetic anhydride at pH 8.0 for 2 h. The fraction of blue membrane was determined from the absorption changes at 660 nm. The experimental points were fitted with two pK_a transitions. In R82K, pK_{a1} = 4.4 (48%) and pK_{a2} = 6.5 (52%). In the WT, pK_a = 4.4 (>95%).

produced by Arg82 in the WT (7.2 − 2.6 = 4.6). This indicates that Asp85 interacts more strongly with Arg82 in the WT than with Lys82 in R82K.

The absorption maximum of the blue form of R82K is close to that in the WT (611 versus 608 nm) and considerably red-shifted compared to that in R82A (586 nm). Thus, the presence of positive charge (Arg82 or Lys82) shifts the absorption maximum of the blue membrane by 22−25 nm to longer wavelengths compared to its position in R82A. The purple form is less sensitive. In R82A the light-adapted form is shifted only 4 nm to the blue (565 versus 569 nm in the WT). The same position is found in R82K (at pH 9).

Two pK_a's of Asp85 in R82K: Evidence for the Interaction of Asp85 with Group X′ and Estimation of the Second pK_a of Asp85 and of Group X′. The pH dependence of the fraction of blue membrane in R82K at low pH (pH <6) can be well approximated by a single transition with pK_a = 3.6. However, at higher pH the experimental points significantly deviate from the simple case described by the Henderson−Hasselbalch equation, indicating that a second acid−base transition with pK_a = 8.0 contributes to the pH dependence of the fraction of protonated Asp85 (see Figure 9B). In order to explain the pH dependence and mechanism of thermal isomerization of the chromophore in bR, we recently proposed a simple model that accounts for the complex titration behavior of Asp85 (Balashov et al., 1993). We suggested that the pK_a of Asp85 depends on the protonation state of another residue X′. When the residue X′ is protonated, the pK_a of Asp85 is low (around 2.6). When X′ deprotonates, the pK_a of Asp85 increases by several pH units. The following scheme represents the coupling of the protonation states of Asp85 and X′ in the ground state (hyphens represent the deprotonated states of the residues; values outside parentheses are for the R82K mutant, and values in parentheses are for the WT):



The pH dependence of the fraction of protonated Asp85 can be described as a function of three parameters (three

pK_a's):

$$f_{DH}(pH) = \alpha/(\alpha + \beta\gamma) \quad (2)$$

where $\alpha = 1 + 10^{(pH-pK_{a3})}$, $\beta = 1+10^{(pH-pK_{a2})}$, $\gamma = 10^{(pH-pK_{a1})}$, pK_{a3} is the pK_a of X′ when Asp85 is protonated, pK_{a2} is the pK_a of X′ when Asp85 is deprotonated, and pK_{a1} is the pK_a of Asp85 when X′ is protonated. The fourth pK_a describing this system, pK_{a4}, is the pK_a of Asp85 when X′ is deprotonated. The four pK_a's are not independent, but rather are bound by the relationship, $pK_{a1} + pK_{a2} = pK_{a3} + pK_{a4}$.

The fit of the experimental curve of f_{D85H} (Figure 9B) with eq 2 gives the following values: $pK_{a1} = 3.7$, $pK_{a2} = 8.0$, $pK_{a3} = 5.7$, and $pK_{a4} = 6.0$, which means that the pK_a of X′ is 8.0 in R82K and that the pK_a of Asp85 shifts from 3.7 to 6.0 upon deprotonation of X′. The pK_a of X′ decreases upon protonation of Asp85 from 8.0 to 5.7. It is interesting to compare the pK_a's of X′ in R82K and WT: when Asp85 is deprotonated, pK_{a2} is 9.7 in the WT and 8.0 in R82K. The difference between pK_{a2} in the WT and R82K is 1.7 pH units, which is the difference in the pK's of free Arg and Lys. This supports the possibility that group X′ is Arg82 in the WT and Lys82 in the R82K (see the following). It should be mentioned, however, that this difference is not observed for pK_{a3} (the pK_a of X′ in the blue membrane where Asp85 is protonated). In the WT pK_{a3} is 4.8 while in R82K it is 5.7; the difference is −0.9. Thus, upon protonation of Asp85, the pK_a of X′ decreases by twice as much in the WT than in R82K (4.9 versus 2.3 units). This corresponds to a similar 2 times larger change in the pK_a of Asp85 upon the protonation of X′, which indicates that the interaction of the negatively charged Asp85 and X′ (presumably positively charged Arg82 or Lys82) in the purple form of the pigments is stronger in the WT than in R82K.

The model of two interacting residues (D85/X′) can be applied to several other mutants. An interesting example of altered purple↔blue transitions is seen in the D85E mutant (Lanyi et al., 1992). D85E has an unusually large amount of blue membrane between pH 4 and 9 (about 70%) and two blue-to-purple transitions with pK_a's of 4.6 and 9.7, which were assigned to two separate equilibria involving two blue and two purple pigments (Lanyi et al., 1992). The pH dependence of the fraction of the purple membrane in the D85E mutant can be fit with the (D85/X′) model under the assumption that the pK_a of Glu85 shifts from 4.6 to 9.6 upon deprotonation of X′. Thus, in the framework of the D85/X′ model, the very high amount of blue membrane at neutral pH in the D85E mutant can be explained by assuming that the second pK_a of Glu85 (when X′ is deprotonated) is 2 pH units higher than that of Asp85 in the WT (9.6 versus 7.5).

The two pK's of Asp85 are not just a peculiarity of the R82K and D85E mutants. In the WT we have observed a complex titration curve of the blue membrane with pK_a's of 2.6 and 9.7 (Balashov et al., unpublished result). The fit of the data indicates that at high pH, when X′ is deprotonated, the pK_a of Asp85 in the WT is ca. 7.5. The two pK_a's of Asp85 provide new evidence for the importance of electrostatic coupling between key amino acids and complex protonation−deprotonation (titration) behavior in bR (Bashford & Gerwert, 1992; Balashov et al., 1993; Sampogna & Honig, 1994).

The dependence of the pK_a of Asp85 on the ionization state of X′ seems to be an important finding that can contribute to understanding not only the ground state properties of bR but also its behavior in the photocycle during light-induced proton transfer from the Schiff base to Asp85 and subsequent proton release. Since Asp85 remains protonated in the M, N, and O intermediates (Souvignier & Gerwert, 1992), its pK_a must be high in these states.

The Nature of the Second Acid Transition. Arg82 is close to both aspartic acid residues and should affect the pK_a not only of Asp85 but also of Asp212. The pK_a of Asp212 is unknown, but it is lower than that of Asp85 since, according to Metz et al. (1992), Asp212 remains ionized when Asp85 is protonated (at pH 2). If the pK_a of Asp212 is sensitive to Arg82 substitutions, one may expect to see a spectral transition at low pH, with pK_a substantially perturbed by R82 mutations. As shown in Figures 3 and 4, at very low pH we did see a shift of the absorption spectrum to the blue and a decrease in extinction. The pK_a of the shift depends on the presence of a positive charge in the 82 position (pK_a = 3.1 in R82A, 1.5 in R82K, and 0 in the WT), which supports the electrostatic nature of this shift and is consistent with the hypothesis that it is due to the protonation of Asp212. This interpretation, however, needs further investigation.

Red Shift of the Chromophore Absorption Band (Alkaline Form of R82K). At high pH, wild-type bR undergoes transformation into an alkaline form, bR_{alk}, which is accompanied by a small (1.5 nm) red shift of the chromophore absorption band, a small red shift of the tryptophan near-UV bands (peaks at 288 and 296 nm), and an increase in absorbance at 238 nm. In the WT, the transition into bR_{alk} occurs with $pK_a \sim 9.0$ (in 167 mM KCl) (Balashov et al., 1991). As shown in Figure 5, R82K undergoes similar changes. The pK_a of the red shift in dark-adapted R82K is 7.7 in 150 mM KCl (Figure 6A). The shift of the Trp absorbtion band shows a similar pK_a. In the WT, the absorption changes due to the chromophore red shift overlap with the transition into a form of bR absorbing at 480−500 nm, P480 (Balashov et al., 1991; Druckmann et al., 1982). In R82K, the red shift of the chromophore absorption band analogous to the bR→bR_{alk} transition in the WT occurs at lower pH, and it is clearly separated from the BR→P480 transition. Thus, the R82K mutation affects the pK_a of the red shift of the chromophore and tryptophan absorption bands, decreasing the pK_a by 1.3 units. This means that the group responsible for the absorption shifts is perturbed by the R82K mutation.

pH Dependence of Light Adaptation. The photoreversibility of light adaptation in R82K (the non-negligible quantum yield for photoisomerization from *trans* to 13-*cis*) is related to alterations of the chromophore binding site. In the WT under normal conditions photoisomerization from *all-trans* to 13-*cis* is inhibited. However, upon solubilization of bR (Casadio et al., 1980) and under dehydrating conditions, in dry films (Korenstein & Hess, 1977; Kouyama et al., 1985) or at high concentrations of glycerol (Balashov et al., 1988), light-induced *trans*→13-*cis* isomerization in the pigment takes place. This phenomenon is also observed in artificial pigments (Steinberg et al., 1991; Brown et al., 1992) and some mutants, such as Y57N expressed in *H. salinarium* (Govindjee et al., 1995) and mutants expressed in *E. coli*: R82Q (Thorgeirsson et al., 1991), D212E, and Y185F (Dunach et al., 1990). There is no well-established mechanism for control of photoisomerization around the $^{15}C{=}N$ bond, which is involved in the formation of 13-*cis*-bR from *all-trans*-bR. Balashov et al. (1988) suggested that a water

molecule, possibly bound to the Schiff base, may be a key factor in preventing photoisomerization around the $^{15}C{=}N$ bond. On the basis of this idea we suggest that the R82K mutation alters hydrogen bonding in the retinal binding site. This is not unexpected since Arg82 (and Tyr57) probably forms hydrogen bonds with water molecules, bound also to Asp212 (Humphrey et al., 1994).

An interesting peculiarity of the R82K mutant is that the photoreversibility is pH-dependent (with $pK_a = 7.7$). At pH 5.6, light adaptation of the mutant produces much less trans pigment than at pH 9.2, where complete transformation of 13-*cis* pigment into *trans* takes place (Figure 7A). We assume that rearrangement of the chromophore environment takes place with $pK_a = 7.7-8.0$, apparently due to the deprotonation of some residue (Lys82 or group X′).

Thermal Isomerization Occurs through the Transient Protonation of Asp85. Studies of the pH dependence of the rate constant of dark adaptation in R82K mutant confirmed the conclusion that Asp85 is the residue that controls the rate of dark adaptation and thus that of thermal isomerization (Balashov et al., 1993). The proposed model of two interacting residues, Asp85 and X′, describes the pH dependence of thermal isomerization in R82K. The proportionality between the rate of thermal isomerization and the fraction of protonated Asp85 (Figure 9) implies that protonation of Asp85 is the key factor in catalyzing thermal isomerization in bR and in determining the pH dependence of this process. We conclude that thermal isomerization, even at high pH, occurs upon transient protonation of Asp85. Protonation of Asp85 reduces the barrier for isomerization at least in part by increasing delocalization of the π-electrons in the chromophore (Warshel & Ottolenghi, 1979; Sheves & Baasov, 1984; Balashov et al., 1993; Song et al., 1993).

Role of Arg82 in the Control of the pK_a of Asp85 and Thermal Isomerization. One of the main motives for studying the R82K mutant was to determine how the change in the intrinsic pK_a of the residue in position 82 would affect the pH dependence of the rate constant of thermal isomerization (as an indicator of the fraction of protonated Asp85 and the pK_a of group X′). If Arg82 is indeed responsible for the decrease in the rate of dark adaptation at high pH ($pK_a = 9.7$ in 150 mM KCl), as discussed in Balashov et al. (1993), then substitution of Arg82 with Lys should shift the pK_{a2} of the pH dependence of the rate constant of dark adaptation to lower pH. This is exactly what was found: pK_{a2} was shifted from 9.7 in the WT to 8.0 in the R82K mutant. The magnitude of the shift (1.7 pK units) coincides with the difference in the pK's of free Arg and Lys in water, assuming that Arg82 and Lys82 are in similar environments in bR. This finding supports the hypotheses that Arg82 is the group controlling the pK_a of Asp85 and that it deprotonates with a $pK_a = 9.7$ (in 150 mM KCl) in the WT. However, one cannot exclude that the correspondence of values of the expected and observed differences in pK_{a2} is coincidental. In order to check this we plan to study some other mutants. At this moment there are two possible explanations for the observed 1.7 unit shift in the pK_a of X′ produced by R82K mutation. Correspondingly, there are two models of the involvement of Arg82 in the control of the pK_a of Asp85.

(1) The most straightforward interpretation is that Arg82 in the WT (Lys82 in R82K) is the group X′; deprotonation of Lys82 in R82K and of Arg82 in the WT is responsible for pK_{a2}. This requires that the pK_a of Arg82 is 9.7 in the WT (in 150 mM KCl). This explanation is in agreement with the pH dependence of dark adaptation in R82A, which is described by only one term with $pK_a = 7.5$. This single $pK_a = 7.5$ can easily be explained by suggesting that the second transition with higher pK_a is missing because the group responsible for it (Arg82) is missing and the pK_a of Asp85 is shifted to ca. 7.5. The pH dependence of the rate constant of dark adaptation of a mutant in which the second group (X′) is absent should be described by only one term, as in R82A. An alternative explanation is that if the R82A mutation causes pK_{a1} (pK_a of Asp85) to become equal or close to pK_{a2} (pK_a of X′), then the pH dependence could appear as if it were caused by only one component.

It should be mentioned, however, that the pK_a of group X′ is sensitive not only to Arg82 mutations. Our measurements of the pH dependence of dark adaptation on the Y57F mutant showed that, in this mutant, the pK_a of X′ (pK_{a2}) is equal to 8.6 (unpublished data). This result suggests that Tyr57 interacts with X′ (since the pK_{a2} is shifted 1 pH unit to lower pH in Y57F). There is evidence that Tyr57 may deprotonate with a pK_a around 9−10 (Govindjee et al., 1992, 1995), but Y57 is not X′ itself since the second pK_{a2} is still present in the pH dependence of k_{da}. These data indicate that X′ should be considered as a cluster of interacting groups (including Arg82, Tyr57, and hydrogen-bonded water molecules).

(2) pK_{a2} in the pH dependence of dark adaptation may not be associated directly with the deprotonation of Arg82 but with some unidentified group X′. The pK_a of this group, however, should depend on the presence of Arg and is sensitive to the substitution of Arg with neutral (R82A) and even charged residues (R82K). From the pH dependencies of the rate constant of dark adaptation, k_{da}, it follows that the pK_a of this group is 9.7 when Arg82 is present (in the WT), 8.0 when Arg82 is substituted with Lys (Figure 9A), and equal to or less than 7.5 or more than 10.5 when Arg82 is substituted by an uncharged residue (in R82A), so that it is not seen in the pH dependence of k_{da} in the pH range between 6 and 11 (Balashov et al., 1993). Since group X′ is sensitive to substitution of Arg82, it should be located close to Arg82. An important feature of group X′ is that it should interact with Asp85 and, upon deprotonation, increase the pK_a of Asp85; conversely, protonation of Asp85 should decrease the pK_a of X′. This interaction may be direct or through Arg82. Arg82, interacting with Asp85 and group X′, may couple these two groups.

It is possible that Arg82 is primarily involved in the control of the pK_a of Asp85, but does not deprotonate. Instead, at high pH, it moves away from Asp85 and thus changes the pK_a of Asp85, as discussed earlier (Balashov et al., 1993). This motion of Arg82 should be controlled by group X′.

Model 2, based on close interaction between Asp85, Arg82, and group X′, can perhaps account for the observed 1.7 pH unit change in the pK_a of X′ (pK_{a2}) in the R82K mutant through alteration of these interactions upon Arg-82→Lys substitution, although this explanation is not as straightforward as assuming that X′ is Arg82 (model 1). On the other hand, changes introduced by the R82K mutation may not be exclusively caused by alteration of the pK_a of the group in position 82. Arg82 may also play a structural role (which is possible taking into account its ability for multiple hydrogen-bonding interactions), and its substitution may cause changes coupled to the alteration of hydrogen bonding rather than changes in pK_a.

Evidence That Arg82 (Lys82 in R82K) Is Both Group X′ and the Proton Release Group. The identity of residue X′ responsible for the change in the pK_a of Asp85 at high pH and the relation of X′ to the proton release group X (Ebrey, 1993; Lanyi, 1993; Zimányi et al., 1992) are intriguing questions. The possible identity of residue X′ was discussed recently (Balashov et al., 1993; Balashov & Ebrey, 1994). Several residues have been proposed as candidates for the group controlling the behavior of bR at high pH: a tyrosine (Kalisky et al., 1981; Balashov et al., 1991), such as Tyr57 or Tyr185, Arg82 (Balashov et al., 1993; Kono et al., 1993), a Lys residue (Maeda et al., 1988), or a carboxyl group (Drachev et al., 1993).

We have proposed (Balashov et al., 1993) that the same group that is responsible for the behavior of bR at alkaline pH's may be the proton release group X (that is, X′ and X are the same group). In the M intermediate (in which Asp85 is protonated) group X has a pK_a = 5.7−5.8 (Zimányi et al., 1992). Mathies et al. (1991) proposed that Arg82 is the proton release group. However, since the pK_a of Arg82 was thought to be high, it was suggested that a water molecule associated with Arg82 (Rothschild, 1992; Braiman et al., 1988) or with Arg82 and Tyr57 (Lanyi, 1993) may be a better candidate. However, the pK_a of water is also very high (15.8 for bulk water), and some special conditions would have to be imposed to decrease its pK_a by 10 pH units. On the other hand, there are examples of very low pK_a, positively charged residues in proteins; for example, the pK_a of a Lys in acetoacetate decarboxylase is 5.9 (Schmidt & Westheimer, 1971).

There are several kinds of evidence suggesting that Arg82 is group X′ (or at least the principal part of a complex of residues constituting group X′). (**1**) Substitution of Arg82 by Lys causes a shift in the pK_a of dark adaptation at high pH (pK_a of X′) by 1.7 pH units (from 9.7 to 8.0). This is equal to the ΔpK of Lys and Arg82, which suggests that residue X′ is Arg82 (in the WT), assuming that this difference in the pK_a's of Arg82 and Lys82 is preserved in the pigments. Most importantly, it suggests that the pK_a of Arg82 is 9.7 in the WT and that the pK_a of Lys82 is 8.0 in R82K. (**2**) Arg82 is located close to Asp85 and apparently interacts directly with the carboxylate of Asp85 in the ground state of bR, forming a salt bridge (Stern & Khorana, 1989; Balashov et al., 1993; Humphrey et al., 1994; Greenhalgh et al., 1991). Removal of the positive charge associated with Arg82 (in the R82A and R82Q mutants) causes a shift in the pK_a of Asp85 from 2.6 to 7.2 (Subramaniam et al., 1990; Balashov et al., 1993; Brown et al., 1993). This is close to what our model predicts for the pK_a of Asp85 when X′ is deprotonated, 7.5. In the R82K mutant the pK_a of Asp85 upon deprotonation of X′ is predicted to be 6.0 (as obtained from the fit of the data shown in Figure 9B), which is close to the value of 6.5 obtained in experiments in which the positive charge of Lys82 was removed by acetylation. (**3**) Group X′ appears to be missing from the R82A mutant as it should be if X′ is Arg82 (see the preceding discussion of pK_a of X′ in R82A, model 1). (**4**) Proton release is drastically delayed in the R82A mutant (Balashov et al., 1993), indicating that the proton release group is missing or nonfunctional in R82A.

On the basis of these arguments, we propose that Arg82 is likely to be group X′ (pK_a = 9.7 in the WT) that is responsible for the alkaline behavior of bR and the control of the pK_a of Asp85. Since the pK_a of group X′ is lowered significantly upon protonation of Asp85, it is likely that it is also the proton release group X, whose pK_a is estimated to be 5.7 when Asp85 is protonated upon M formation (Zimányi et al., 1992). It should also be noted, however, that several residues in the counterion complex strongly interact with each other (De Groot et al., 1990). Group X′ could in fact be a cluster of hydrogen-bonded residues and water molecules that all contribute to the pK_a of X′ (and X). Thus, Tyr57 affects the pK_a of X′ and proton release (Lanyi, 1993; Govindjee et al. 1995; Balashov, unpublished result). According to Humphrey et al. (1994), there is a water molecule bound to Tyr57 and Arg82. One may speculate that protonation of Asp85 leads to the neutralization of Arg82 not through direct deprotonation of this residue, but rather through the association of a hydroxide ion, which leads to the release of a proton from a water molecule as suggested by Braiman et al. (1988). Some reservations should be mentioned. Brown et al. (1993) inferred the value for the pK_a of Arg82 in the WT as 13.8 rather than 9.7 which, if correct, rules out the possibility that Arg82 is identical to X′ and X. In this case, an alternative interpretation can be given: rather than deprotonating, Arg82 moves away from Asp85 at high pH, thus decreasing the electrostatic interaction between them; this motion could be controlled by the deprotonation of yet another group. Scharnagl et al. (1995) have recently suggested that E204 is the proton release group.

Light-Induced Schiff Base Deprotonation and M Formation. Role of the pK_a *of Asp85.* We found that, unlike the WT and similar to the R82A mutant, M formation in R82K is fast (1.5 μs) and pH-independent in the pH range between 5 and 11. In the WT the rise time decreases from 80 μs at low and neutral pH to 6 and 0.3 μs at pH 10 (Liu, 1990). In order to explain the increase in the rate of M formation in the R82A mutant, we suggested that the removal of a positive charge near Asp85 (from position 82) caused a drastic increase in the pK_a of Asp85 and an increase in the rate of light-induced proton transfer from the Schiff base to Asp85 in the L to M transition (Balashov et al., 1993). Although this mechanism still seems reasonable, the result found in the R82K mutant does not directly support it, since we would expect a slower rate of M formation at low pH where Lys82 (or the X′ group) is expected to be protonated. This suggests that in the case of R82K the electrostatic interaction of Asp85 with the charge on Lys82 is not the rate-limiting factor. Instead, the ΔpK_a between the Schiff base and Asp85 in R82K favors protonation of Asp85 (M_1 state). This means that the pK_a of Asp85 is high enough in L to allow fast Schiff base deprotonation.

It is possible that relatively small changes in the pK_a of Asp85 strongly affect the kinetics of Schiff base deprotonation. In several cases where fast M formation is observed, the pK_a of Asp85 in the ground state is higher than that in the WT at neutral pH. Some examples are as follows: WT at pH 10, where the pK_a of Asp85 shifts from 2.6 to 7.5, R82A and R82Q (where the pK_a of Asp85 is 7.5), Y57F (pK_a = 4.9) (Govindjee et al., 1995), and D85E (the pK_a of Glu85 is 4.6) (Lanyi et al., 1992). This may also be the case in R82K. The difference in the pK_a's of Asp85 in R82K and in the WT is 1 pH unit. The M intermediate is formed rapidly in R82K, with a rise time of 1.5 μs. If we assume that deprotonation of the Schiff base rather than proton transfer between the Schiff base and Asp85 is the rate-limiting step, then, from the rate constant of Schiff base deprotonation, we can estimate that the pK_a of the Schiff base is about 3−4 in the intermediate L. This estimate is

obtained by using a simple relationship between the pK_a and the rate constants of deprotonation, k_d, and proton association, k_a: p$K_a = -\log(k_d/k_a)$, assuming that $k_d = 10^6$ s^{-1} (from $\tau_d = 1$ μs) and $k_a = 10^{10}-10^9$ M^{-1} s^{-1} (Gutman & Nachliel, 1990; El-Sayed, 1993) for the environment of the Schiff base, which includes several water molecules. Let us assume that the pK_a of the Schiff base is 3 and that the pK_a of Asp85 does not change in L (equal to 3.6). Under these conditions, 80% of the molecules will convert into M with fast 1.5 μs kinetics and come into equilibrium with the remaining 20%, which will stay in L until some other process increases the relative affinity of Asp85 for protons (for example, increase in the pK_a of Asp85 due to the deprotonation of X′).

In the WT at neutral pH, the ΔpK_a in the L intermediate is initially unfavorable for Schiff base deprotonation, and the amplitude of very fast M (time constant 1.7−2.7 μs) is only 6−11% (Heberle et al., 1993; Cao et al., 1995). This can be attributed to a less favorable ΔpK_a between the Schiff base and Asp85. (Let us say that the pK_a of the Schiff base is 3.4 while the pK_a of Asp85 is 2.6.) The unfavorable difference in pK_a of about −0.8 unit will shift the equilibrium toward L. Thus, a ΔpK_a for the L→M transition of about 1.4 units can account for the different kinetic behavior of R82K and WT. This difference might be associated with the different pK_a's of Asp85 in the two pigments. The main phase of M rise in the WT occurs with a time constant of about 85 μs, which should be connected with subsequent changes in the environment (and pK_a) of the Schiff base and Asp85. The deprotonation of group X′ will further increase the pK_a of Asp85 and stabilize the proton on Asp85. It could be that two groups are involved in proton transfer to the surface, which can explain the two subsequent phases in the M rise (Heberle et al., 1993; Cao et al., 1995). This picture is in general agreement with the model of two (or three) sequential M intermediates (Varo & Lanyi, 1991; Ebrey, 1993; Lanyi, 1993; Brown et al., 1994b). It suggests that the apparent kinetics of M rise is strongly affected by the ΔpK_a between the Schiff base and Asp85. When the proton affinity of Asp85 is high (high pK_a of Asp85), which is the case in the WT at high pH, R82A, and presumably R82K, the apparent kinetics of M rise reflects the rate constant of Schiff base deprotonation, while in the cases where the initial ΔpK_a between the Schiff base and Asp85 is unfavorable (WT at neutral pH), accumulation of M is controlled by the processes that increase the proton affinity of Asp85, one of which is presumably the deprotonation of X, which increases the pK_a of Asp85. This, however, is not the only possible interpretation of the kinetics of light-induced Schiff base deprotonation, and other models based on the conformational heterogeneity of the bR population cannot be disregarded. The preceding hypothetical picture emphasizes the role of the pK_a of Asp85 and changes in its environment (including group X′) in proton transfer in bR. However, several other factors may be involved in the catalysis of Schiff base deprotonation in bR.

Theoretical calculations of Scheiner and Duan (1991) and model experiments of Gat and Sheves (1993) show that mutual orientation of donor and acceptor groups (most likely mediated by water molecule(s)) might be an important factor controlling the pK_a of the Schiff base, and presumably the pK_a of Asp85 as well. Another factor is the distance between the Schiff base and Asp85. A change in the distance by 1 Å may be enough to alter the rate of proton transfer by several orders of magnitude (Scheiner & Hildebrand, 1985; Gutman & Nachliel, 1990). The polarity of the environment of the Schiff base and Asp85 (dielectric constant) is also an important factor (Scheiner & Duan, 1991; Balashov et al., 1993).

The changes produced by the R82A and R82K mutations promote fast M formation, but inhibit (completely in R82A and partially in R82K) fast proton release from the X group by somehow uncoupling the deprotonation of the group X from the protonation of Asp85 in the L→M transition. An alteration of the interaction between Asp85 and proton release group X (and X′) resulting in an increased pK_a of Asp85 seems a likely explanation for the fast M rise.

The exact cause of fast M rise in R82A and R82K mutants still has to be established. It could be a higher pK_a of Asp85, a slightly closer distance between the Schiff base and Asp85, a more favorable alignment of these two groups (Brown et al., 1994b), or a lower dielectric constant, which destabilizes the Schiff base−Asp85 ion pair and favors stabilization of the proton on Asp85. In any case, it results in a more favorable ΔpK between the Schiff base and Asp85 for the M intermediate.

Proton Transfer in R82K. The transient light-induced proton release and uptake detected with pyranine in the bulk solution show a kinetic pattern similar to that seen in the WT at pH 7 (release precedes uptake), which provides evidence that Lys82 supports fast proton release in R82K. The signal in R82K is different from that seen in R82A and R82Q, where the release is drastically delayed (rise time of about 30 ms) and occurs after the uptake (Otto et al., 1990; Balashov et al., 1993; Brown et al., 1994a). The latter was interpreted as an inhibition of the function of the proton release group, which normally (in the WT at neutral pH) releases a proton upon protonation of Asp85 in the L-to-M transition. The proton in R82A apparently is released in the last step of the photocycle (during reformation of initial bR) from D85, directly or through intermediate groups, as proposed for WT bR at low pH (Cao et al., 1993; Lanyi, 1993).

The appearance of the WT-like transient pyranine signal in R82K indicates that substitution of the positive charge of Arg82 with that of Lys allows the function of the fast proton release pathway, but with lower efficiency than the WT. The transient pyranine signal, plotted for the same amount of M, is only 15% of that in the WT, indicating that the efficiency (measured as the number of protons released within 2 ms per M formed) is low (15% compared to that in the WT). Under continuous illumination the proton transfer efficiency of R82K is at least 40% of that in the WT, as indicated in measurements of light-induced pH changes in vesicles containing R82K. From comparison of the relative amplitudes of the transient (pyranine) and steady state proton signals of R82K, it follows that, most likely, only a small fraction of protons (15−30%) is released rapidly from R82K, while most of the protons are released on the same time scale as proton uptake. The two processes cancel each other and are not seen in the transient signal in suspensions of membranes.

The lower efficiency of Lys82 in the catalysis of proton release compared to Arg82 can be explained in the framework of the D85/X′ model. It is likely that proton release group X and residue X′ affecting the rate of thermal isomerization are in fact the same residue (or group of interacting residues), but in different states (X′ is the ground state, while X corresponds to the M intermediate in which

the Schiff base is deprotonated). Deprotonation of the Schiff base in M presumably causes a shift in the pK_a of X′ of about 1 pH unit in the WT, which follows from the comparison of the pK_a of X′ when Asp85 is protonated (4.8) and the pK_a of group X in M [5.8, according to Zimányi et al. (1992)]. By assuming that in R82K the deprotonation of the Schiff base causes a similar shift in the pK_a of X′, one would expect a pK_a of group X for R82K of about 6.7 (5.7 + 1.0). Thus, at pH 6.7, only about half of the R82K molecules in the M state can rapidly release a proton from X, which partially explains the small amplitude of the pyranine signal in R82K.

The explanation of the lower efficiency of the proton release group in R82K may also be given in a framework of indirect involvement of Arg82 in the proton release (not as a proton release group but as a group strongly affecting the proton release pathway and the pK_a of the proton release group). Lys is slightly (about 1 Å) shorter than Arg82, so that its charge (and a proton of the amino group) may not exactly be in the "correct" position. This change in the position of the positive charge may affect the pK_a's of other groups participating in the proton transfer.

Another factor is that the presence of a guanidinium group, in which the positive charge is delocalized over a larger space, may be crucial for interactions with other key residues and water molecules in the proton release channel. According to the structure of Humphrey et al. (1994), one amino group of Arg82 interacts with the carboxylate of Asp85 while the other amino group interacts with two water molecules, themselves bound to Tyr57 and Asp212. The amino group of Lys may be an insufficient substitute for the guanidinium group of Arg and may not effectively support the hydrogen-bonding network in the proton release channel. In this case, however, one has to conclude that the slower proton release pathway is less sensitive to R82K mutation (since the overall proton efficiency of R82K is at least 40% that of the WT, while only 15% of the protons are released fast).

The exact role of Arg82 in proton release still needs to be established. The simplest scheme is direct involvement through its light-induced deprotonation as a consequence of M formation (Mathies et al., 1991; Balashov et al., 1993; Balashov & Ebrey, 1994) or through deprotonation of a water molecule associated with Arg82 (Braiman et al., 1988) and probably Tyr57 (Lanyi, 1993).

Accessibility of Lys82 (in R82K) for Modification by Acetic Anhydride: Effect on the pK_a of Asp85. In R82K, incubation with acetic anhydride results in a shift of the pK_a of the purple-to-blue transition to 6.5. In the WT under similar conditions, the pK_a of the purple-to-blue transition was increased by only 1.8 units from 2.6 to 4.4 (Figure 14), but no blue membrane was seen at pH 6.5. This result provides evidence that the high pK_a of the purple-to-blue transition in modified R82K (pK_a = 6.5) is due to the acetylation of Lys82 (which is missing from the WT). Thus, even though Lys82 is apparently buried inside the protein, it is still accessible to such reagents as acetic anhydride in the ground state. These data also show that the pK_a of Asp85 is about 6.5 when the positive charge on Lys82 is removed by acetylation, which is close to the pK_a of Asp85 at high pH (when X′ is deprotonated) obtained from the fit of the pH dependence of the fraction of blue membrane (pK_a = 6.0; see the legend to Figure 9B). This result provides additional evidence that Lys82 may be the X′ group in R82K.

In summary, the mutant of bR in which Arg82 was replaced with Lys (R82K mutant) was constructed and expressed in *Halobacterium salinarium*. (a) The pK_a of the purple-to-blue transition (pK_a of Asp85) is ca. 3.6 in the dark-adapted R82K. It is closer to that in the WT (2.6) than to that in R82A (7.2), indicating that the pK_a of Asp85 is indeed controlled by the positive charge on Arg82 (or Lys82). (b) The pH dependence of the fraction of blue membrane (fraction of protonated Asp85) shows two acid−base transitions in R82K, which is explained by the strong interaction of Asp85 and a residue X′ that deprotonates at high pH (pK_a = 8.0). Deprotonation of X′ causes a shift in the pK_a of Asp85 to 6.0 at high pH. (c) The pH dependence of the rate constant of dark adaptation coincides with the pH dependence of the fraction of protonated Asp85. This indicates that transient protonation of Asp85 is the key factor and intermediate step in the catalysis of thermal isomerization of the chromophore in bR. (d) The pK_a of the rate constant of dark adaptation is shifted by 1.7 units to lower pH from 9.7 in the WT to 8.0 in R82K, suggesting that Lys82 in R82K (and Arg82 in the WT) may be group X′ controlling the pK_a of Asp85 and the rate constant of thermal isomerization at high pH (or at least the principal part of X′) and is also part of the proton release complex. (e) The shape of the transient pyranine signal is similar to that of the WT (proton release precedes uptake). However, the H^+/M ratio is only about 15% of that in the WT. Under steady state illumination, R82K incorporated in vesicles pumps protons with an efficiency at least 40% that of the WT. (f) The rate of M formation is fast (1.5 μs) in R82K and does not depend on pH. (g) Several pH-dependent processes in R82K show a pK_a close to 7.5−8.0 (in 150 mM KCl): photoreversibility of light adaptation, yield of M intermediate, and a small red shift in the chromophore and tryptophan absorption band (which is also observed in the WT with pK_a = 9.0). (h) Lys82 is accessible to modification by acetic anhydride. The pK_a of Asp85 is ca. 6.5 in acetylated R82K, which is in agreement with the model suggesting that deprotonation of Arg82 in the WT and of Lys82 in R82K causes a shift in the pK_a of Asp85.

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