Effect of the Arginine-82 to Alanine Mutation in Bacteriorhodopsin on Dark Adaptation, Proton Release, and the Photochemical Cycle†

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ABSTRACT: The pH dependence of the rate constant of dark adaptation (thermal isomerization from *all-trans*- to 13-*cis*-bR) drastically changes when Arg82 of bacteriorhodopsin is replaced by an alanine. In the wild type (WT) the rate decreases sharply between pH 2.5 and pH 5. In R82A the sharp decrease is shifted to pH >7. This correlates with the shift in the p*K* of the purple-to-blue transition from pH 2.6 in the wild type to pH 7.2 in the mutant (in 150 mM KCl). We propose that the same group that controls the purple-to-blue transition, namely, Asp85, catalyzes dark adaptation. The rate of dark adaptation in the R82A mutant is proportional to the fraction of protonated Asp85, indicating that dark adaptation occurs when Asp85 is transiently protonated. Thermal isomerization is at least 2×10^3 times more likely when Asp85 is protonated (blue membrane) than when it is deprotonated (purple membrane). The pH dependence of dark adaptation in the WT can be explained by a model in which the rate of dark adaptation in the WT is also proportional to the fraction of protonated Asp85 and that the p*K* of Asp85 depends on some other group, X, which deprotonates (or moves away from Asp85) with p*K* 9 and causes the shift in the p*K* of Asp85 from 2.6 to 7.2. The quantum yield of light adaptation is at least an order of magnitude less in R82A as compared to the WT. The rise time of M formation is very fast in R82A and, unlike the WT, pH independent (1 μs versus 85 and 6 μs in the WT at pH 7 and 10, respectively). The activation energy of the L to M transition is 6.9 kcal/mol versus 13.5 kcal/mol in the WT. Thus the loss of a positive charge in the active site greatly increases the rate of light-induced deprotonation of the Schiff base. In the R82A mutant, the M decay at pH >8.8 is much faster than the recovery of initial bR, which suggests a decrease in the rate of back-reaction from N to M. In a suspension of R82A membranes the rate of proton release as measured by the pH-sensitive dye pyranine is delayed by at least 20-fold (in 2 M KCl), while the uptake of protons did not change much (12 ms in the WT versus 8 ms in R82A). This suggests that Arg82 is associated with proton release in bR. A simple electrostatic model involving interaction between charged groups provides an interpretation for the shift of the p*K* of Asp85 in the R82A mutant, change in the rate constant of M formation, and pH dependence of the rate constant of dark adaptation.

Studies of variants of bacteriorhodopsin (BR)[1] produced by site-directed mutagenesis have revealed several key amino acids which are involved in proton transfer and in maintaining the functional state of the pigment [for reviews see Mathies et al. (1991), Lanyi (1992), Oesterhelt et al. (1992), Rothschild et al. (1992), and Ebrey (1993)]. These studies, together with a moderate resolution structure for bR (Henderson et al., 1990) have led to the conclusion that the Schiff base interacts with Asp85, Asp212, Arg82, probably Tyr57, Tyr185, and a divalent cation [see the above reviews and Jonas and Ebrey (1991)]. It was concluded that Asp85 is a part of a "complex" counterion (de Groot et al., 1990) and acts as the primary acceptor of protons in the process of light-induced proton transfer. Moreover, when Asp85 is protonated at low pH, the color of the membrane changes in the so-called purple-to-blue transition [see reviews and Metz et al. (1992)].

Several properties of bacteriorhodopsin are altered when Arg82 is mutated to another amino acid. (1) When expressed in *Escherichia coli* mutants of bR where the positively charged Arg82 is replaced by neutral alanine or glutamine show a drastic shift in the p*K* of the purple-to-blue transition from pH 2.8 to pH 6.5–7; thus at neutral pH about half of the mutant pigment is in the blue form, which does not show an M intermediate and is inactive in proton transfer (Stern & Khorana, 1989; Subramaniam et al., 1990; Drachev et al., 1992). (2) At pH 7.5 the R82Q pigment, when incorporated into phospholipid vesicles, pumps protons with a diminished efficiency as compared to the wild type (Stern & Khorana, 1989; Miercke et al., 1991). Transient proton release and uptake from the R82Q pigment was minute; a small transient proton uptake signal was observed in R82A, the decay of which correlated with absorption changes at 650 nm (Otto et al., 1990). (3) Dark adaptation was found to be much faster in R82A than in the wild type, but the light minus dark difference spectrum looked like that of the purple-to-blue transition (Dunach et al., 1990). The 13-cis isomer was found in the light-adapted state of R82Q (Lin et al., 1991a; Thorgeirsson et al., 1991). (4) The rate of M formation in the R82Q mutant was found to be close to that in controls

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[1] Abbreviations: bR, bacteriorhodopsin; DA, dark adapted; LA, light adapted; PC, phosphatidylcholine; WT, wild type.

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(Drachev et al., 1992) or faster (Lin et al., 1991b).

These studies were done on proteins which were produced by expression of the synthetic gene of bR in *E. coli* (Khorana, 1988). The pigment (e-bR) was obta' ed by subsequent reconstitution of the apoprotein with retinal in lipid vesicles. This pigment has slightly altered features compared to bR expressed in *Halobacterium halobium*: the absorption maximum is shifted to shorter wavelengths, the pigment is less stable and has a narrower pH range of proton pumping, and the rates of the photocycle reactions are altered. Some features of the pigment having mutations at R82 remain controversial or unclear. These include (i) the effect of the mutation on dark adaptation, the cause of the fast dark adaptation rate at neutral pH (Dunach et al., 1990), and the presence of the 13-cis isomer in the light-adapted state; (ii) the kinetics of M formation and decay and its pH dependence; and (iii) correlation of proton transfer with reactions of the photocycle.

Recently, a synthetic bR gene has been expressed into *H. halobium* (Ni et al., 1990; Needleman et al., 1991), so that mutant pigments could be studied in their native lipid environment. In this paper the R82A mutant in purple membrane isolated from *H. halobium* was examined with respect to transient proton release and uptake kinetics, light and dark adaptation processes, and photochemical features.

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 pT7Blue vector (Novagen, Madison, WI) containing PCR-generated fragments. All *E. coli* strains were grown in 2× YT or LB media (Ausubel et al., 1989). The wild-type and R82A *bop* genes were expressed in *H. halobium* IV-8 (a gift of Richard Needleman) which contains a stable IS*H1* insert in the *bop* gene (DasSarma et al., 1984). *H. halobium* IV-8 was grown on C media (Needleman et al., 1991).

Site-Directed Mutagenesis of BR. Oligonucleotide-directed site-specific mutagenesis was performed essentially as described by Menick (1991). The 2.7-kb *Bam*HI–*Hin*dIII *bop* gene insert was restricted from the *H. halobium* shuttle vector pMC-1 (a generous gift of R. Needleman) and ligated into M13mp19. Arg82 was replaced with Ala by using the mutagenic primer 5′CTGG GCG GCG TAC GCT GAC 3′, which contained the indicated two-nucleotide mismatch. Mutants were initially screened by colony blot hybridization and then plaque purified, and the mutation was verified by dideoxy sequencing (Sanger et al., 1977). 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. halobium IV-8. The R82A BR mutant and wild-type control were transformed into the *H. halobium* strain IV-8 which contains and IS*H1* insert within the *bop* gene. Transformation was performed as described (Lam & Doolittle, 1989; Ni et al., 1990; Needleman et al., 1991). Briefly, IV-8 cells were grown to mid-log phase, pelleted, and resuspended in spheroplasting buffer (2 M NaCl, 27 mM KCl, 50 mM Tris-HCl, pH 8.75, 15% sucrose), which was then brought to 85 mM EDTA. A total of 200 μL of spheroplasts was gently transferred to a tube containing 10 μg of plasmid DNA. After 10 min 220 μL of filter-sterilized 60% PEG600 (in 40% spheroplasting buffer) was added, and the solution was gently mixed. The mixture was incubated for 15 min; then 1 mL of regeneration salt buffer (Needleman et al., 1991) was added. The spheroplasts were pelleted by centrifugation, resuspended in growth media (plus 15% sucrose; Needleman et al., 1991), and incubated at 37 °C for 48 h. Aliquots were plated on growth media agar plates with 15% sucrose and 5 mg/mL mevinolin (a generous gift of Merck Sharp & Dohme). Several independent clones were picked (after 2 weeks of growth) and struck out on growth media plates; single colony isolates were grown in 2 L of growth media to characterize each BR mutant.

Southern and Sequence Analysis of the R82A Mutant. Southern blot analysis was carried out as described (Ausubel et al., 1989). Genomic DNAs from *H. halobium* cells IV-8 and IV-8 cells transformed with wild-type or the R82A *bop* mutant were digested with *Pst*I, transferred to Genescreen Plus membranes, and hybridized with a ^{32}P-labeled 500-bp *Kpn*I *bop* gene fragment. Additionally, the R82A *bop* gene was amplified by polymerase chain reaction from the R82A transformed IV clones. PCR was performed with 1 μg of sheared *H. halobium* genomic DNA as template (Saiki, 1990) utilizing a sense primer, 5′-CGGTGCGGATCCGACGT-GAAGATGG-3′, which corresponds to a sequence of 435 base pairs 5′ of the *bop* start codon, and an antisense primer, 5′-GGGCTGTCA*AGC*TT*GTGCGATC-3′, corresponding to sequences immediately 3′ of the *bop* gene stop codon (contains two mismatches to generate a unique (*Hin*dIII site). The reaction was run on a DNA thermal cycler (Perkin-Elmer Cetus) as follows: cycle 1, 5 min at 94 °C, 2 min at 55 °C, and 2 min at 70 °C; cycles 2–25, 1 min at 94 °C, 2 min at 55 °C, and 2 min at 70 °C. The 1150-bp PCR product was gel purified, ligated into pT7Blue (Novagen, Madison, WI), and sequenced.

Isolation of Purple Membrane. Purple membrane was prepared from *H. halobium* IV-8 transformed with wild-type and R82A *bop* as described (Oesterhelt & Stoeckenius, 1974).

Sample Preparations. To maintain the sample pH, the following buffers were used: Mes, Mops, Hepes, Tricine, Ches, and Caps (Good et al., 1966). The pH was adjusted by 0.1–1 N solutions of HCl and KOH. Egg phosphatidylcholine vesicles containing WT and R82A bacteriorhodopsin were obtained as described in Govindjee et al. (1980). The pH in the suspensions of membranes and light-induced changes of the pH in liposomes were measured with a pH electrode (Radiometer America Inc.). The p*K* of the purple-to-blue transition and the rates of dark adaption in the wild type (strain S9) were measured in polyacrylamide gels in order to prevent aggregation (Mowery et al., 1977). The purple membrane containing gels were prepared as described previously (Liu et al., 1991). For light adaptation a 500-W slide projector was used in combination with cutoff and heat filters (glass and solution of $CuSO_4$). Samples were illuminated at 500–550 nm for 10–20 min at 20 °C.

Spectroscopic Methods. Absorption spectra were recorded in 5-mm quartz cuvettes thermostated at 20 °C using a Cary-Aviv 14 spectrophotometer (Aviv Associates, Lakewood, NJ) by digital measurement with 1-nm steps and a spectral bandwidth of 1 nm. Kinetic measurements of dark adaptation were done at 580 nm using the kinetic mode of the spectrophotometer. A homemade cryostat was used for low-temperature measurements (Balashov et al., 1991).

Flash-induced absorption changes were measured on a kinetic spectrophotometer (Dancshazy et al., 1986; Govindjee et al., 1990). The actinic pulse was from a Quanta Ray DCR-11 Nd:YAG laser (532 nm, 7 ns, 5–10 mJ/pulse; Spectra

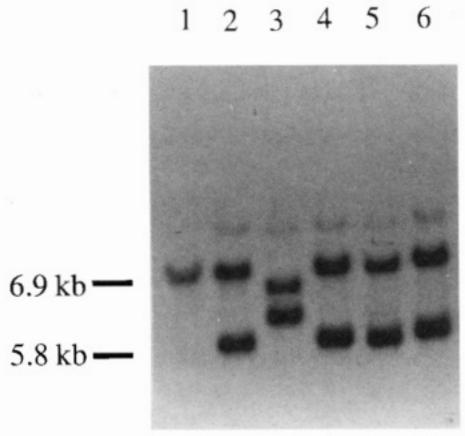


FIGURE 1: Southern blot analysis of wild-type and R82A transformants. *H. halobium* IV-8 chromosomal DNA was digested with *Pst*I, separated on a 1% agarose gel, transferred to Genescreen Plus, and hybridized with a ^{32}P-labeled 500-bp *bop Kpn*I fragment. Lane 1 contains DNA from *H. halobium* IV-8 (*bop*::IS*H1*); lanes 2–4 contain DNA from independent clones from *H. halobium* IV-8 transformed with the wild-type *bop* gene; lanes 5 and 6 contain DNA from independent clones of *H. halobium* IV-8 with the R82A *bop* mutant.

Physics, Mountain View, CA). Flash-induced pH changes were measured at pH 7–7.2 using the pH-sensitive dye pyranine.

RESULTS

The pMC-1 plasmid containing the wild-type or R82A *bop* gene can exist as an autonomously replicating form or integrated into the *H. halobium* chromosome. Importantly, the plasmid-born wild-type or mutant *bop* gene recombines at the *bop* chromosomal locus, or expression of the original chromosomal *bop* gene could occur by excision of the IS*H1* element. Therefore, Southern analysis was performed to ensure that the IS*H1* element interrupting the chromosomal *bop* gene of strain IV-8 has not been excised and that no obvious recombination events have occurred between the plasmid-born and chromosomal *bop* genes. Southern analysis of chromosomal DNA demonstrates that the R82A mutants and all but one of the wild-type transformants have maintained the chromosomal *bop*::IS*H1* (Figure 1). *H. halobium* IV-8 has a single 6.9-kb *Pst*I fragment that corresponds to the chromosomal *bop* gene containing the 1.1-kb IS*H1* insert (Figure 1, lane 1). The 6.9-kb fragment which hybridizes to *bop* is present in two of the three wild-type (lanes 2 and 4) and both R82A (lanes 5 and 6) transformants. The transformants also contain the 5.8-kb *Pst*I fragment that corresponds to the plasmid *bop* gene. Clearly the plasmid *bop* gene from one wild-type *bop* transformant (lane 3) has recombined at the *bop* chromosomal locus (lane 3). This clone was discarded. Importantly, the R82A *bop* gene was amplified by polymerase chain reaction to ensure that the transformed R82A *bop* gene was fully intact and the only sequence changes were those we engineered. Sequencing of the entire 1150-bp PCR fragment revealed that the only changes to the *bop* gene sequence changed R82 to an A (data not shown).

The Blue-to-Purple Transition in R82A bR. When the pH in the dark was increased from 6.0 to 9.0, the absorption maximum of R82A mutant membranes shifts from 585 to 554 nm (Figure 2A). The difference absorption spectra "pH 6 minus pH$_i$" have maxima at 620 nm (Figure 2B). They do not show any noticeable changes in the protein absorption bands at 280 nm (data not shown). The pK of the transition between the blue and purple forms, determined from the

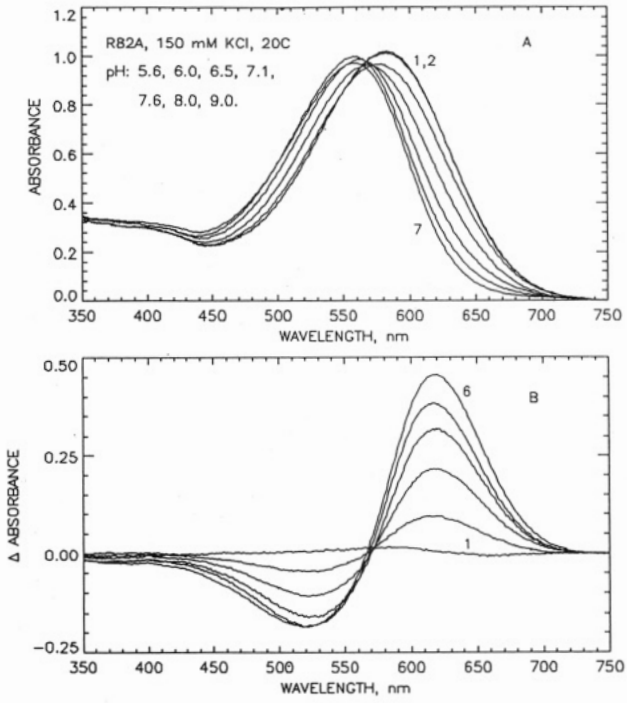


FIGURE 2: Blue-to-purple transition in the R82A mutant. (A) Absorption spectra of the suspension of the R82A mutant membranes in 150 mM KCl at pH$_i$. Curves 1, pH 5.6 (10 mM Mes); 2, pH 6.0 (8 mM Mes); 3, pH 6.5 (10 mM Mes); 4, pH 7.1 (5 mM Mops); 5, pH 7.6 (8 mM Hepes) 6, pH 8.0 (7 mM Tricine); 7, pH 9.0 (7 mM Ches). In all cases the samples were prepared as follows: to the initial 420-μL unbuffered dark-adapted suspension of membranes was added 5 μL of buffer. pH and absorption spectra were measured at 20 °C. (B) Difference absorption spectra at pH 5.6 – pH$_i$. Maxima in the difference absorption spectra are at 617–620 nm.

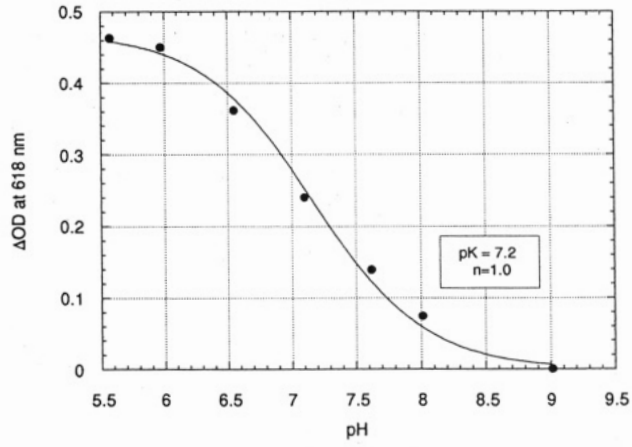


FIGURE 3: pH dependence of absorption changes at 618 nm in the blue-to-purple transition in R82A membranes in 150 mM KCl at 20 °C. The solid line is the best fit titration curve with pK_a 7.20 and n = 1.04.

absorbance changes at 620 nm, is 7.2 in 150 mM KCl (Figure 3), which is close to the value reported previously for the e-bR mutant, 6.9 (Subramaniam et al., 1990). The shape of the titration curve (Figure 3) indicates that only 1 H$^+$ per bR is involved in the transition (n = 1). The formation of blue bR involves the protonation of Asp85, and according to the ^{13}C NMR data it is the only internal aspartic acid that is protonated in this transition (Metz et al., 1992). In the WT the pK_a of the transition is 2.6 in 150 mM KCl (n = 1.5). The shift of the pK of the purple-to-blue transition to 7.2 in the case of R82A indicates that in the wild type the positive charge on Arg82 causes a shift in the pK of Asp85 by 4.6 units (from 7.2 to 2.6), allowing a majority of the pigment to be in the

functionally active purple form at pH >4 in the WT.

Spectral Transitions at Low pH: Blue bR′ and Acid Purple Form of R82A. Below pH 4 the blue form of the R82A pigment (blue bR) undergoes additional transformations in the dark. In the absence of Cl^- ions a decrease of pH to 2 causes a 13-nm blue shift (from 587 nm at pH 4 to 574 nm at pH 2) and a decrease of absorption by 20% (measured in gels). The species formed at pH 2 has a broad spectrum and bluish color, similar to the blue bR present between pH 6 and pH 4. It can be called blue-bR′ following the nomenclature of Varo and Lanyi (1989). In 1 M Na_2SO_4 blue bR′ is formed with a pK around 3.

In the presence of Cl^- ions (2 M KCl or NaCl) an acid purple species is formed, analogous to that in the wild type (Fischer & Oesterhelt, 1979; Mowery et al., 1979). Its formation is accompanied by an additional blue shift from 574 to 566 nm, an increase in maximal absorbance, a decrease of absorption in the red (at wavelengths >600 nm), and a narrowing of the absorption band. The absorption maximum of the acid purple is at 566 nm (at pH 1 in 2 M KCl). Contrary to other low-pH species it does not absorb significantly at >660nm. At shorter wavelengths the acid purple species of R82A has characteristic β-bands at 426, 396, and 369 nm, which are present in the spectrum of the light-adapted R82A and are indicative of the trans-isomer form of the pigment. The pK of formation of acid purple species of R82A is 1.9 in 0.2 M NaCl and 1 M Na_2SO_4. In 0.8 M NaCl and 1 M Na_2SO_4 two transitions associated with acid purple formation can be seen with pKs of 3.5 and 2. The first pK is close to the pK of blue bR′ formation, which suggests that acid purple is formed from this species rather than directly from the blue bR. The pKs of acid purple formation in R82A are shifted 1 unit to higher pH compared to the wild type, for which two acid purple forms with pKs of 2.8 and 1 (in 0.8 M NaCl + 1 M Na_2SO_4) were described (Varo & Lanyi, 1989). Thus the pK of the acid purple form is much less affected by R82A mutation compared to the pK of blue bR.

The acid purple species of R82A is formed at significantly lower pH compared to the blue bR. This suggests that protonation of at least one or even more groups (other than Asp85) is a prerequisite for acid purple formation. This group should be close enough to Asp85 to modulate its ability to bind chloride ions. An interesting possibility is that it is Asp212. The negative charges on the surface of the membrane also apparently control the formation of acid purple species (Fischer & Oesterhelt, 1979).

Alkaline Forms and Alkaline Denaturation of R82A Membranes. For R82A, increasing the pH from 9.0 to 11.0 in the dark causes a decrease in absorbance at 566 nm and an increase in absorbance at 460 nm (Figure 4A). This transition is similar to the formation of the alkaline form of the WT which absorbs at 480 nm (Balashov et al., 1991). However, in R82A a larger amount of the pigment undergoes this transition (30% versus 14% in the WT at pH 11, measured from the absorption decrease at 640 nm). This is shown in Figure 4B, where the difference spectra "pH 11 – pH 9" for R82A and WT are shown. The absorption spectrum of the alkaline species of R82A has a broad absorption maximum at 480–540 nm (Figure 4A, curve 3) rather than at 480 nm, as in the WT (Balashov et al., 1991), suggesting the presence of two alkaline products, one absorbing at ca. 460 nm and the other at 540 nm. The larger amount of the alkaline species in the mutant compared to the WT indicates that Arg82 is important not only in maintaining the functional stability of the pigment at low pH (by controlling the purple-to-blue

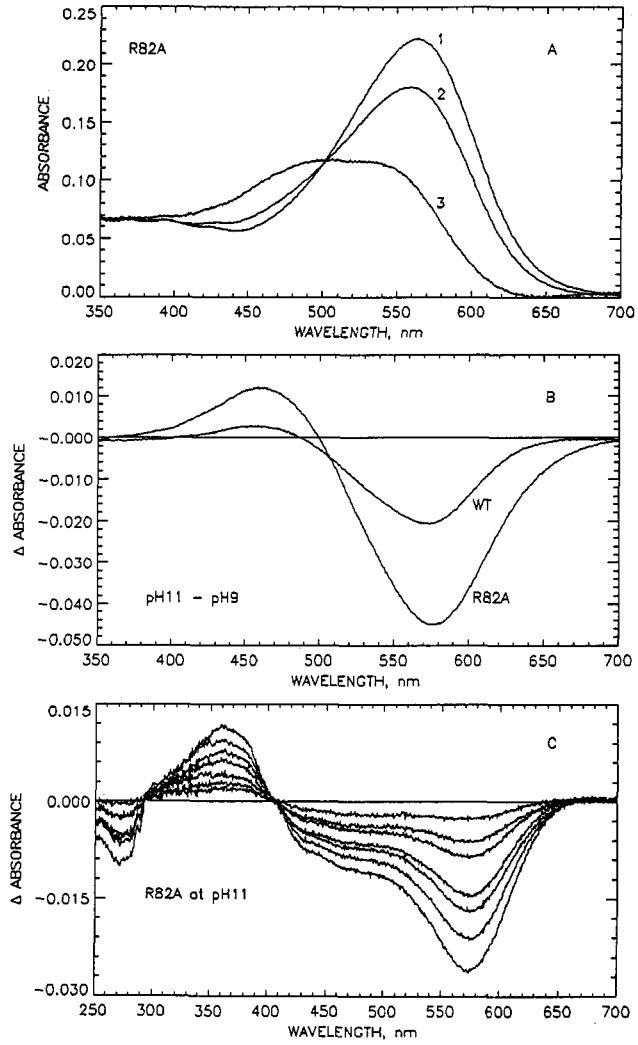


FIGURE 4: Absorption changes in R82A at high pH. (A) Curves: 1, absorption spectrum of light-adapted bR at pH 9.0; 2, the spectrum after the pH was adjusted (in the dark) to 11.0 with 17 mM Caps; 3, the spectrum of the alkaline form calculated from the difference spectrum of curve 2 minus curve 1 and its fraction approximately equal to 0.30 (determined from the absorbance decrease at 640 nm). (B) Difference spectra of absorbance changes poduced by increasing the pH from 9.0 to 11.0 in R82A (curve 2 minus curve 1 in panel A) and in the WT (the initial ODs of both samples are the same at the maximum). (C) Absorption changes upon incubation of R82A in the dark at pH 11 for 4, 8, 12, 22, 26, 34, and 45 h. All samples contained 150 mM KCl at 20 °C.

transition) but also at alkaline pH. In R82A we do not observe the red shift of the chromophore absorption band which was observed in the WT (pK around 9 in 170 mM KCl) (Balashov et al., 1991). The amplitude of absorption changes at 287 and 297 nm due to the shift of the tryptophan absorption band at pH 9 is approximately 2 times smaller in R82A than in the WT. The peak at 244 nm which is apparently due to deprotonation of a tyrosine residue (Balashov et al., 1991) appears at pH >9.5 (p$K \approx$ 10.5 in 150 mM KCl).

Upon prolonged incubation (45 h) at pH 11.0 in the dark, a band at 362 nm appears (Figure 4C), which apparently indicates alkaline denaturation. Alkaline titration (in the presence of 0.02% Triton X-100) indicates that in R82A the pK of the formation of the 362-nm species is shifted 0.7 pH unit to lower pH as compared to the WT.

Light and Dark Adaptation in R82A. Illumination of the R82A dark-adapted (DA) membranes with 500–560-nm light at pH 8.8 causes a red shift of the absorption spectrum and an increase in the maximum extinction (Figure 5A). Com-

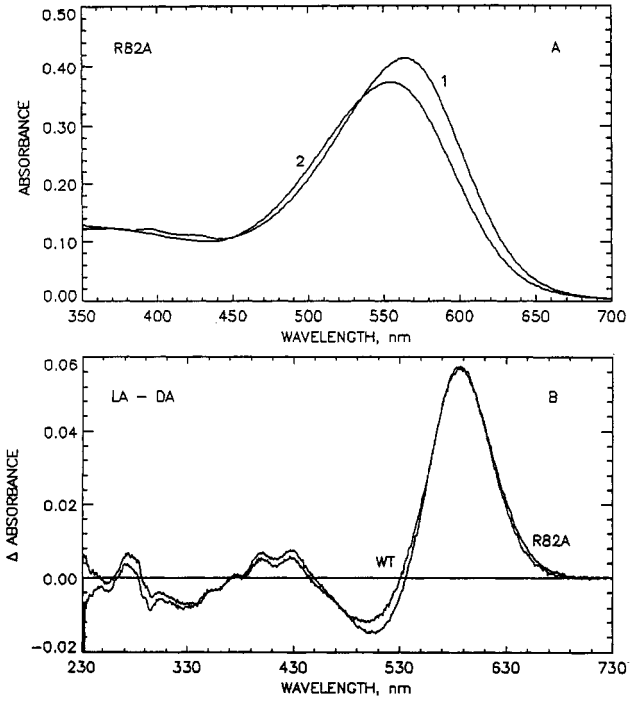


FIGURE 5: (A) Absorption spectra of light-adapted (1) and dark-adapted (2) R82A membranes in 150 mM KCl at pH 8.8, 20 °C. (B) Difference absorption spectra of light adapted minus dark adapted in the R82A mutant and WT.

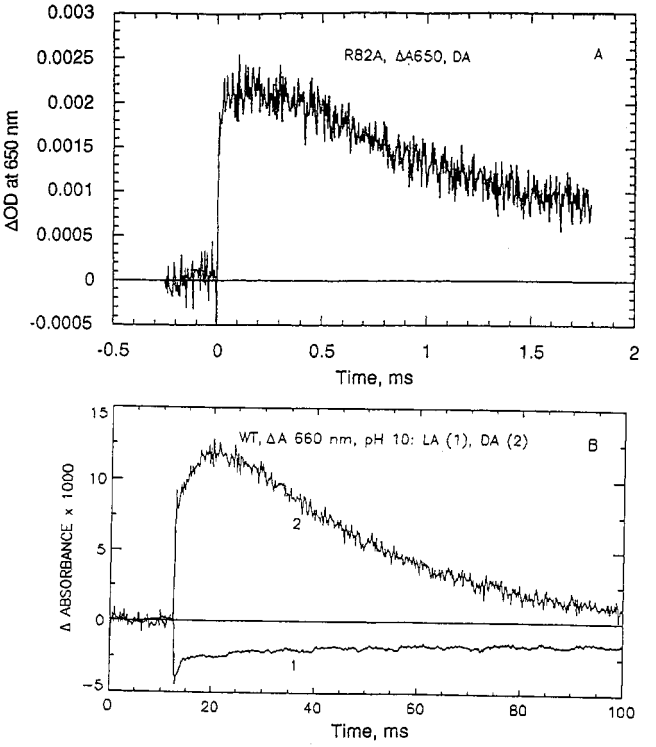


FIGURE 6: Formation and decay of the bathoproduct of 13-*cis*-bR (monitored at 650–660 nm) in the dark-adapted membranes in the R82A (A) and WT (B) at 20 °C. In the WT, absorption changes were measured at pH 10 in both the light-adapted (1) and dark-adapted (2) membranes. In order to prevent light adaptation of the sample, only a single trace was taken on each sample. In R82A (pH 8.9), only the trace taken in the DA state is shown (see Figure 7 for the absorbance changes in the LA state).

pared to the absorption maxima in the WT, the absorption spectra of both light-adapted (LA) and DA states of R82A (at pH 8.8) blue shift 4–6 nm: 565 nm (versus 569 nm) for the LA state and 555 nm (versus 560 nm) for the DA state.

Dunach et al. (1990) found that light adaptation of R82Q and R82A e-bR mutants showed abnormal features. The "light minus dark" difference spectrum at pH 8 was similar to the difference spectrum of the purple-to-blue transition caused by a decrease in pH. Illumination at pH 8.5 caused the appearance of a photoproduct absorbing at 470 nm. We find that light adaptation of the R82A mutant expressed in *H. halobium* is different from that described for the mutant expressed in *E. coli*. At pH 8 and above, R82A from *H. halobium* showed normal light and dark adaptation processes. The difference absorption spectrum "light adapted minus dark adapted" (Figure 5B) in the mutant is very close to that in the WT and different from the difference spectrum of the purple-to-blue transition. As for the WT, the spectrum has a maximum at 586 nm and minimum at 505 nm (due to 13-cis to all-trans isomerization). In the near-UV region (300–430 nm) there is a group of β-bands which reflect the same transition. In both cases (R82A and WT), light adaptation causes an increase in absorption in the region of tryptophan and tyrosine absorption bands (at 270–290 nm) but no significant changes at 240 nm, suggesting no change in the protonation state of a tyrosine residue during dark adaptation. However, at pH 7.2 the maximum in the light minus dark difference spectrum shifts toward longer wavelengths (600 nm), which can be caused by the light-induced purple-to-blue transition, a phenomenon described by Dunach et al. (1990). This phenomenon, however, is not unique to the R82A or Y185F mutant. Wild-type bR shows similar behavior at pH values near its purple-to-blue transition p*K* (Balashov et al., unpublished results). Apparently, it is caused by different p*Ks* for the purple-to-blue transition in *trans*-bR and 13-*cis*-bR.

Prolonged illumination of R82A membranes at pH 6 with red light causes the transformation of the blue form of the pigment into pink species (maximum in the absorption spectrum is at 484 nm) similar to that formed in the wild type at pH 2. The pink species is stable in the dark but converts back to the blue membrane under blue light illumination. Thus in R82A, a bistable reversible photochromic transition occurs at pHs closer to neutral.

The Bathoproduct K^C and Isomeric Ratios of the Chromophore in the LA and DA States. Quantum Yield of Light Adaptation. In the DA state, the R82A mutant shows a rapid flash-induced increase in absorbance at 650 nm (Figure 6A); since this is not seen in the LA state (Figure 7B), it must be due to the formation of the bathoproduct of 13-*cis*-bR, K^C (this bathoproduct in the wild type has also been referred to as ^{610}C (Sperling et al., 1977)). The lifetime of K^C in R82A is more than an order of magnitude shorter compared to the wild type (1 ms at pH 8.8 and 2.1 ms at pH 10 vs 30 ms in the WT at pH 10, 17 mM CHES). In the dark-adapted wild-type membrane the flash-induced absorption increase at 650-660 nm is biphasic: a fast (unresolved) phase is accompanied by a slower additional increase in absorbance (half-rise time is approximately 2 ms; see Figure 6B). This process can be ascribed to a transformation of K^C into a product which, by analogy with the trans cycle, may be called KL^C. A similar but faster increase in absorbance at 650 nm is observed also in the R82A mutant (Figure 6A). This rise of absorbance at 650 nm was observed also in the R82A mutant expressed in *E. coli* (Otto et al., 1990) and was attributed to L or early O formation (of the trans photocycle). However, the present data indicate that these absorbance changes at 650 nm are probably associated with the 13-cis photocycle rather than with the trans cycle.

The isomer ratios in the LA and DA state of R82A were determined from the flash-induced absorbance changes at

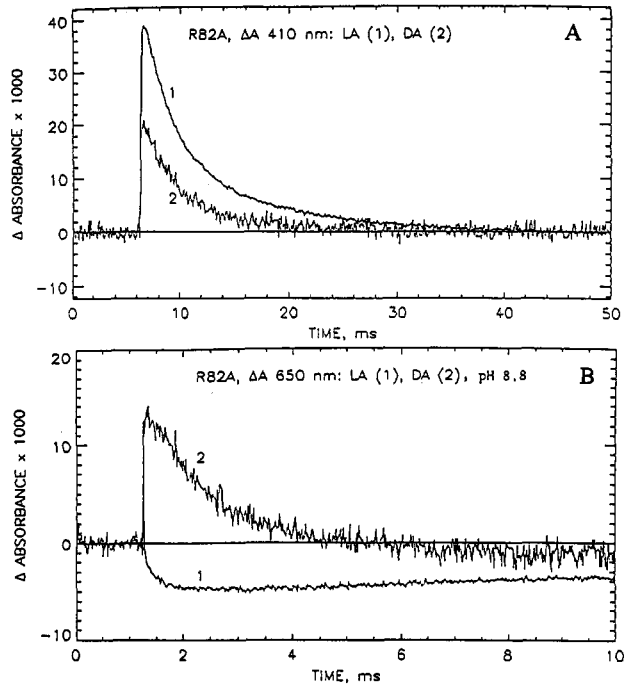


FIGURE 7: Light-induced absorption changes in LA (1) and DA (2) R82A membranes at 410 nm (A) and 650 nm (B), pH 8.8 and 20 °C.

410 nm (M formation) and 650 nm (formation of K^C) at pH 8.8 (Figure 7). As noted above, the fast light-induced increase in absorbance at 650 nm is absent in the LA state, suggesting that 13-*cis*-bR is absent in the LA state of the mutant. In the DA state, the amount of M is 50 ± 2% of that in the LA state (Figure 7A). These data indicate that there is 50 ± 2% *trans*-bR in the DA state and close to 100% (>98%) in the LA state.

The isomer ratio is pH dependent in dark-adapted R82A. The fraction of *trans*-bR is higher at pH 6 (in the blue form) than at pH 9 (in the purple form). This was demonstrated in the following experiment. The sample was dark adapted at pH 6 (in 1 mM Mes and 0.5 mM Na_2SO_4) so that no further changes occurred. Then the pH was increased to 9 in the dark (10 mM Ches + 4 mM Na^+), which turned almost all of the pigment into purple form. Subsequent incubation in the dark resulted in absorption changes which were similar to those observed during usual dark adaptation of light-adapted samples. The amplitude is approximately half of that observed when the sample was fully light adapted at pH 9. This indicates that the fraction of all-trans pigment in the dark-adapted blue membrane at pH 6 was about 25% larger than at pH 9, and thus the all-trans pigment comprises about 75% of the blue membrane while the equilibrium fraction of *trans*-bR at pH 9 was 50%. The pH dependency of the isomer ratio in dark-adapted bacteriorhodopsin in the region of the purple-to-blue transition gives evidence for different p*Ks* of Asp85 in *trans*- and 13-*cis*-bacteriorhodopsin.

Using absorption changes at 650 and 410 nm as a measure of the fraction of 13-cis and trans isomers, we find that in the WT (pH 8.8) a few flashes (532 nm, 7-ns laser pulses, 5 mJ/cm^2) are enough to produce partial light adaptation while in the mutant (at the same pH) 10–12 times more flashes are needed to produce a similar effect. From this we conclude that the quantum yield of light adaptation is at least an order of magnitude less in R82A compared to the WT. In the WT, it is 10 times less than the quantum yield of the primary light reaction (Balashov & Litvin, 1981), which is about 0.64 (Govindjee et al., 1990). Thus in the mutant the quantum yield of light adaptation is less than 0.006. It is interesting

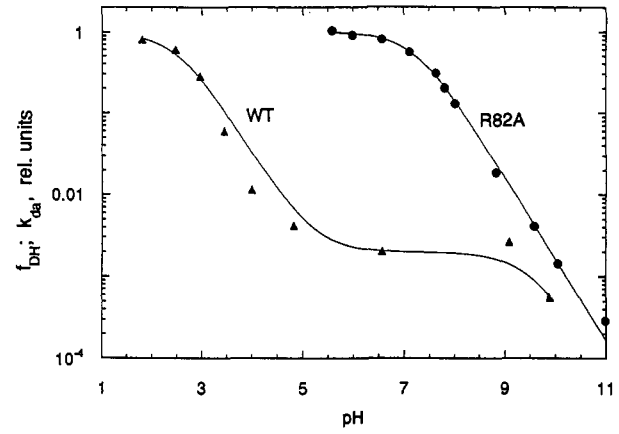


FIGURE 8: pH dependence of the rate constant of dark adaptation, k_{da}, in R82A (circles) and WT (triangles) and the fraction of protonated D85, f_{DH} (solid lines) in 150 mM KCl at 20 °C. The rate constants are scaled to 1 at their maximal values. The fraction of protonated D85 (fraction of blue membrane) for the R82A mutant was obtained from the pK plot in Figure 3 fitted by the Henderson–Hasselbach equation in the following form: $f_{DH} = 1/10^{pH-pK_a}$, where $pK_a = 7.2$. The maximum value of k_{da} in R82A (at pH 5.5) was determined to be 4.5×10^{-3} s^{-1}, which corresponds to the half-time of dark adaptation $t_{1/2} = 100$ s. The maximal rate of dark adaptation in the WT (at pH 1.4) was 10 times faster, 4.0×10^{-2} s^{-1}. The fraction of protonated D85 (or blue membrane) for the WT was obtained from the equation $f_{DH} = \alpha/(\alpha + \beta\gamma)$, where $\alpha = 1 + 10^{pH-pK_1}$, $\beta = 1 + 10^{pH-pK_2}$, and $\gamma = 10^{pH-pK_3}$. The fit was obtained with $pK_1 = 4.9$, $pK_2 = 9.5$, $pK_3 = 2.6$, and $k^0_{da} = 0.04$ s^{-1} (see Discussion for details).

that the decrease in the quantum yield of light adaptation in the mutant correlates with the decrease in the lifetime of KL^C (^{610}C).

pH Dependence of the Rate of Dark Adaptation in R82A. The pH dependence of the rate of dark adaptation of R82A brings some insight on the mechanism of thermal isomerization of the chromophore in bR and the role of Asp85 in this process. The rate of dark adaptation of the R82A mutant is very pH dependent at pH >7 (Figure 8). The log of the rate constant of dark adaptation, k_{da}, decreases almost linearly with an increase in pH from 7.8 to 10.5 (10 times per pH unit). At pH 6 the half-time of dark adaptation is about 100 s (150 mM KCl, 20 °C). At pH 7.8 the half-time is 7 min (in the WT it was 122 min). At pH 8.8 the half-time is 75 min, at pH 9.6 it is 5.6 h, and at pH 10.0 it is 16 h. This dependence of k_{da} on pH is very different from that in the WT, in which the rate is maximal at pH around 2. Between pH 2.5 and pH 5 the log of the rate decreases almost linearly. At 5 < pH < 9 the rate is almost unchanged, while at pH >9 a further decrease in the rate is observed.

A comparison of pH dependency of k_{da} shows that the pH at which the sharp decrease in k_{da} starts shifts from ca. pH 2.5 in the WT to pH >7 in the mutant (in 150 mM KCl). This shift in pH dependence of k_{da} correlates with the shift in the pK of the purple-to-blue transition from pH 2.6 in the WT to pH 7.2 in the mutant, indicating that dark adaptation is catalyzed by the same group as the purple-to-blue transition, Asp85. The sharp decrease in the rate (10 times per pH unit) indicates that the rate is proportional to the fraction of protonated Asp85 and that transient protonation of Asp85 is necessary for thermal isomerization of the chromophore from all-trans to 13-cis. In full agreement with this, the pH dependence of the rate of dark adaptation, k_{da}, in the R82A mutant was fitted by a simple equation:

$$k_{da}(pH) = k^0_{da} f_{DH} \quad (1)$$

where $k^0_{da} = 4.5 \times 10^{-3}$ is the maximal rate of dark adaptation when Asp85 is fully protonated and f_{DH} is the fraction of protonated Asp85, which can be calculated from the pK of Asp85 (equal to 7.2) using a Henderson–Hasselbach equation:

$$f_{DH} = 1/(1 + 10^{pH-pK}) \quad (2)$$

As shown in Figure 8, both k_{da} and f_{DH} decrease 2×10^3 times from pH 6 to pH 11, which indicates that thermal isomerization is at least 2×10^3 times more likely when Asp85 is transiently protonated (blue membrane) than when it is deprotonated (purple membrane). From this, it follows that the activation energy barrier for thermal isomerization of the chromophore in bR produced by the negative charge on Asp85 is at least 4.5 kcal/mol. Equation 1 quite nicely approximates the rate of dark adaptation in the pH range 5.5–10.5. At pH 11 alkaline denaturation becomes the major source of time-dependent changes of the absorption spectra (Figure 4C). Nevertheless, it was possible to deconvolute the difference spectra in Figure 4C into two components corresponding to (a) denaturation (bleaching of the main chromophore absorption band and appearance of the 362-nm band) and (b) dark adaptation and to estimate the rate of dark adaptation at pH 11. It is 8 times less than at pH 10, which is close to the factor of 10 observed at lower pH.

Thus the shift in pK of the purple-to-blue transition (and so apparently the pK of Asp85) from pH 2.6 to pH 7.2 correlates with the analogous shift of the pH dependency of the rate of dark adaptation. Moreover, the pH dependence of the rate constant of dark adaptation is proportional to the amount of transiently protonated Asp85. This suggests that isomerization involves transient protonation of Asp85; protonated Asp85 catalyzes dark isomerization in bR.

Alterations in the Photochemical Cycle of R82A. The absorption maximum of the M intermediate in R82A is shifted 5–6 nm to the blue as compared to the WT (measured at 20, −70, and −160 °C). At neutral pH the rise of M (at 20 °C) is very fast in R82A; this is similar to the kinetics observed at high pH in the WT. In R82A about 70% of M is formed with a rise time of 1.0 μs and the remaining 30% with 25 μs. In the WT the rise time of M formation is 85 μs at neutral pH and 0.3 μs plus 6 μs at pH 10 (Liu, 1990). In contrast to the WT, in the mutant the rate of M formation does not change in the pH range between 7 and 10.1 (Figure 9). The activation energy of the L to M transition is 6.9 kcal/mol in R82A, which is significantly less than in the WT [13.5 kcal/mol at pH 6.5, 5 mM $CaCl_2$ (Liu, 1990); similar values for the WT were reported in Varo and Lanyi (1991) and Lozier et al. (1992)].

The yield of M is pH dependent in R82A (Figure 10A), correlating with the amount of the purple form of the pigment. At pH 4.3 there is almost no purple form, and there is no M. At pH 8.8 the blue form is absent, and the yield of M is equal to that of the WT. This equality indicates that the photochemical yield of M in the purple form of R82A is close to that in the wild type, 0.64 [see Govindjee et al. (1990)], assuming the extinction coefficients of the pigments and their M's are the same.

M and N Decay and the N to M Back-Reaction. Between pH 7 and pH 8, M decay of R82A is monoexponential with a lifetime of 5 ± 0.5 ms (Figure 10A). At pH 9–10 the recovery of initial absorbance at 570 nm slows down drastically, apparently due to a decrease in the rate of N to bR conversion. In the WT about 50% of M decays fast at high pH while the other half decays with much slower kinetics, which approximately correlates with the recovery of 570-nm absorbance.

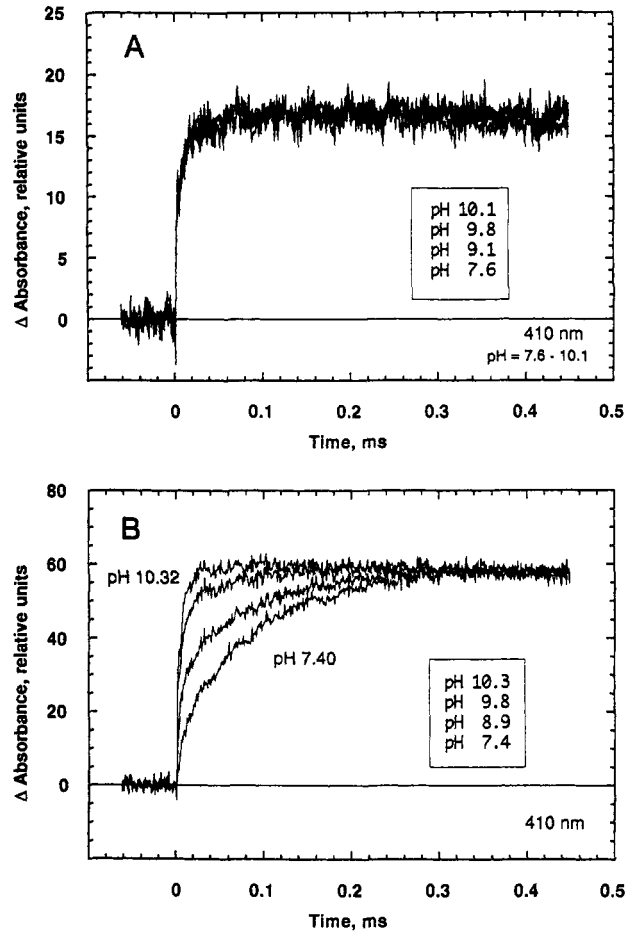


FIGURE 9: Kinetics of M formation in R82A (A) and in the wild type (B) at different pH (curves are normalized at the maximum).

The appearance of a slow component in M decay at high pH in the WT is attributed to an efficient back-reaction from N to M (Otto et al., 1989). As shown in Figure 11, the light-induced absorption change at 410 nm decays much faster than the absorption at 580 nm recovers. This probably indicates that the rate of back-reaction from N to M is much less than the forward one. In the R82A mutant we did not observe an O intermediate (defined as a long-wavelength species arising after M), probably because the rate of the O to bR transition is very fast (Figure 10C).

Proton Release and Uptake in R82A: Suspensions of R82A Membranes. Figure 12 shows the flash-induced proton changes as measured by the transient absorption changes of the pH indicator pyranine in suspensions of WT and R82A purple membranes at pH 7.2. The fast (less than 1 ms) decrease in absorbance of the dye observed in the WT corresponds to the release of protons from the purple membrane. Subsequent restoration of absorbance (rise time 12 ms) corresponds to the uptake of protons by the purple membrane. In 2 M KCl the proton signal from R82A is about 40% that of the WT. The signal from R82A has a positive (uptake) phase (rise time 8 ms) followed by a slow relaxation phase (H^+ release) with a decay time of about 30 ms. Thus in the R82A mutant the order of proton release and uptake is reversed compared to the wild type; i.e., uptake precedes release, which is in agreement with the observation of Otto et al. (1990) made on a mutant expressed in *E. coli* However, kinetically the signal observed in the R82A mutant expressed in *H. halobium* is very different from that described for the mutant expressed in *E. coli*.

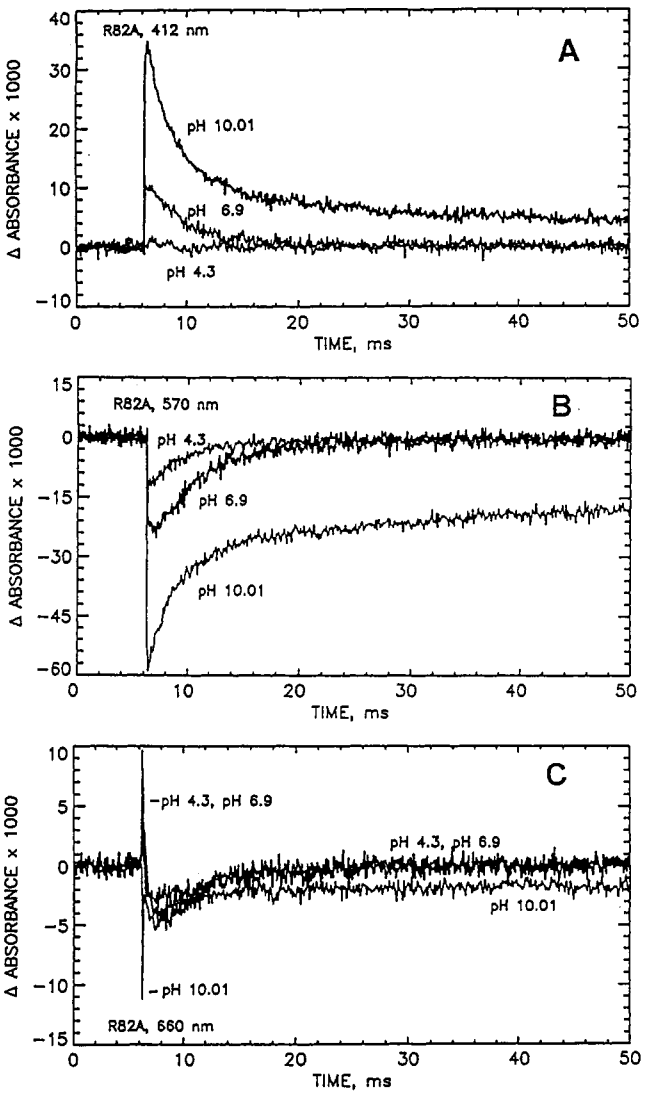


FIGURE 10: The light-induced absorbance changes in R82A at 412 nm (A), 570 nm (B), and 660 nm (C) at pH 4.3, 6.9, and 10.01 at 20 °C (150 mM KCl).

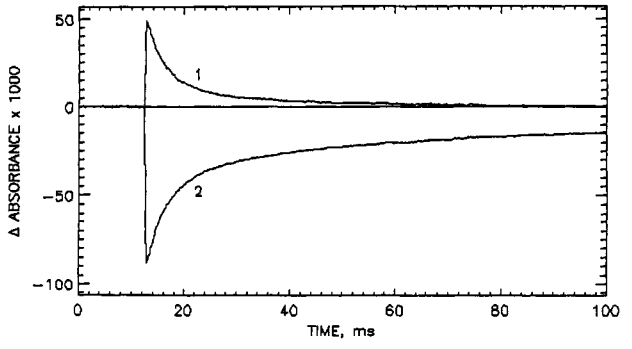


FIGURE 11: Kinetics of flash-induced absorption changes in R82A measured at 580 nm (1) and 410 nm (2) at pH 9.15 (20 mM Ches and 150 mM KCl).

Experiments with liposomes (see below) show that the mutant pumps protons with an efficiency at least 40% of that of the WT. This indicates that proton release is not inhibited in the mutant but highly delayed.

Part of the pyranine signal may be associated with the 13-cis cycle rather than trans cycle. Since the rate of dark adaptation is high at pH 7.2–7.3, some 13-cis pigment should always be present in the sample. In fact, measurements of Otto et al. (1990) on dark-adapted R82A in Chaps micelles

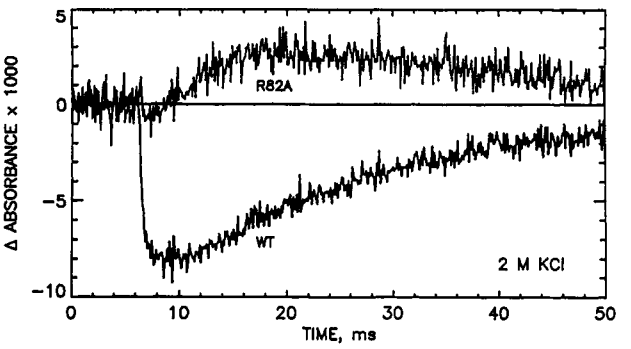


FIGURE 12: Flash-induced transient absorption changes of pyranine at 460 nm in the suspensions of WT and R82A purple membrane in 2 M KCl, pH 7.3. The dye signals are normalized to the same amount of M formed. The decrease in absorbance corresponds to proton release.

show a correlation of the 1-ms pyranine signal (corresponding to release of protons) with the decay at 650 nm, which was interpreted as an L or early O intermediate. Although we find a similar lifetime for these absorption changes (see Figure 6A), our data indicate they should rather be associated with the bathoproduct of 13-*cis*-bR, which has a decay time of about 1 ms in the R82A mutant (see Figure 6A). In our case, due to the very different time course we observe for the pyranine signal (30 ms instead of 1 ms), we think that the 30-ms release phase is not associated with the decay of the bathoproduct of the 13-cis isomer (KL^C).

Thus the lack of a positive charge in the vicinity of the Schiff base in the R82A mutant results in an almost 100-fold increase in the rate of M formation and Schiff base deprotonation (1 μs versus ca. 85 μs) and a more than 30-fold delay in proton release from the membrane (30 ms vs less than 1 ms when measured with pryanine). This suggests that R82 is on the release pathway for protons from the membrane and probably directly catalyzes proton release.

Phospholipid Vesicles Containing R82A: Flash-Induced Proton Changes. Figure 13 shows the kinetics of the light-induced pyranine absorption changes in a suspension of phosphatidylcholine vesicles containing the wild-type or R82A pigments. Three kinetic components can be seen in the pyranine response in the WT vesicles (Figure 13A): (1) One is a small, initial absorbance decrease, which corresponds to the proton release. The half-time of this signal is less than 400 μs; its amplitude is only 8% of the total signal. Apparently, this phase is due to proton release from bR molecules having their N-terminal sides oriented toward the outer surface of the vesicle. (2) Another is a large absorption increase, due to proton uptake (92% of the signal, half-time is 14 ms) by the pigment molecules incorporated into liposomes with their cytoplasmic (C-terminal) side facing outside the vesicle. This orientation is usual for this type of vesicle preparation (Lozier et al., 1976; Govindjee et al., 1980). The amplitudes of the first and the second phase indicate that 92% of the total pigment has inverted (as compared to the cell membrane) orientation in the vesicle. (3) The third phase is a relaxation of the signal (back-flow of protons from vesicles) which is insignificant on the 200-ms time scale. The half-time of the decay can be estimated as ≥2 s.

The light-induced pyranine signal from R82A vesicles, either in 150 mM or in 2 M KCl, also has three phases (Figure 13B,C): (1) The first is due to the uptake of protons. The half-rise time is 11 ms, which is a little faster than the analogous phase in the WT (14 ms). It is reasonable to suggest that the uptake phase, as in the case of the WT, is produced by the

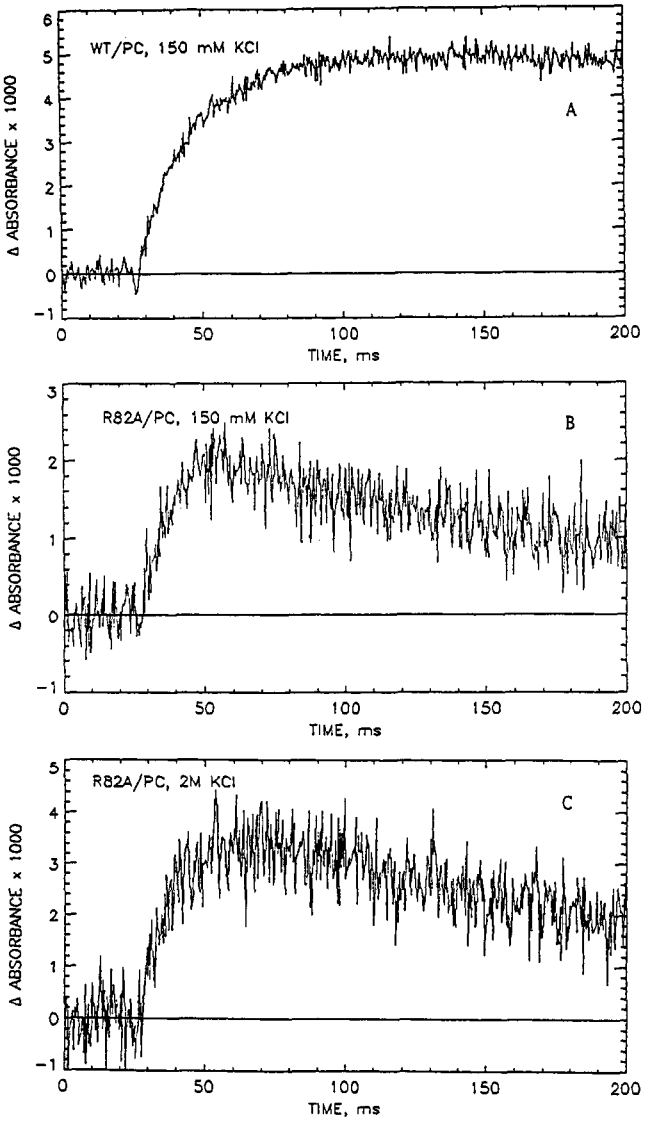


FIGURE 13: Pyranine absorption changes in a suspension of phosphatidylcholine vesicles at 460 nm. The positive signal corresponds to proton uptake. (A) Wild-type containing vesicles in 150 mM KCl, pH 7.3. The amplitude of absorption changes associated with M formation at 410 nm was 16.5 mOD. (B and C) R82A vesicles in 150 mM KCl, pH 7.4 (B), and in 2 M KCl, pH 7.3 (C). The amplitude of the M signal at 410 nm was 13.5 mOD in 150 mM KCl and 24.0 mOD in 2 M KCl.

molecules pumping protons into the liposome. (2) The uptake phase is followed by a slow (half-time 50 ms) phase with opposite sign. The experiments with the WT in vesicles show that this is unlikely to be due to protons leaking out of the vesicles because this leakage occurs on a time scale of seconds (see above), and so this phase most likely corresponds to the 30-ms release phase seen in the suspension of the R82A membranes (see, e.g., Figure 12B). It can be interpreted as a proton release from molecules pumping protons from the vesicle. The deconvolution of the signal into two phases gives values for the fraction of the first and second phases, 68% and 32%, respectively, which apparently reflect the fractions of molecules pumping protons inside and outside vesciles. (3) The third phase is relaxation of the signal with a half-decay time approximately the same as in the WT vesicles (1.5 s) due to the leaking of protons across the vesicle membrane.

Addition of 2 M KCl resulted in an almost 2-fold increase in the amplitude of the pyranine signal compared to that in 150 mM salt (Figure 13C). The amount of M formed is also doubled. This effect can be explained by the salt-dependent

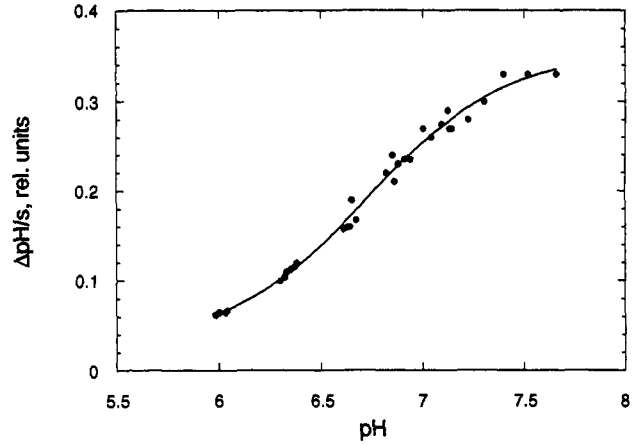


FIGURE 14: pH dependence of the initial rate of light-induced proton uptake in PC vesicles containing R82A in 2 M NaCl under continuous illumination (dots). Curve fit, pK 6.7.

shift of the pK of the blue-to-purple transition so in 2 M KCl at pH 7.2 there is almost twice as much of the purple form than in 150 mM KCl. After normalization of the signal to the amount of M in the WT, the amplitude and kinetics of the signal in 2 M KCl almost coincide with the signal in 150 mM KCl (compare panels B and C of Figure 13). This indicates that the purple form of the pigment is the source of the proton signal and that the efficiency of proton pumping of the purple form in 150 mM KCl and 2 M salt is the same.

An estimate based on deconvolution of the signals into release and uptake phases and estimation of degree of misorientation indicates that the purple form of R82A pumps protons with efficiency almost equal to that of the WT. From the amplitudes of uptake and release phases, one can estimate that orientation of the pigment is much higher in the WT vesicles (92 – 8 = 84%) than in R82A vesicles (68 – 32 = 36%).

Light-Induced pH Changes in R82A Liposomes under Continuous Illumination. Continuous illumination at 500–550 nm caused proton uptake by a suspension of phospholipid vesicles containing R82A or WT, which indicates that the pigment in both cases has a net inside out orientation (pump protons inside the vesicles). The slopes of the initial pH changes caused by continuous illumination of R82A-containing vesicles in 2 M salt are highly pH dependent in the range from 6 to 7 (Figure 14). At pH 6 the signal is very small, while in the WT it is quite large. The increase in the initial rate of proton pumping in R82A vesicles has a pK of about 6.7 in 2 M salt, which roughly coincides with the purple-to-blue transition in the mutant. A second factor which may decrease the proton pumping efficiency of the R82A at neutral and acid pH is the high rate of dark adaptation.

The relative proton pumping activity of R82A vesicles is 0.41 of that of the WT vesicles. The relative efficiency was calculated as the light-induced pH change per amount of pigment: $\Delta pH/A_{max}$. The decrease in proton pumping in R82A vesicles is apaprently caused by a much larger misorientation of the R82A in the vesicles. We find that, according to the flash measurements, the degree of orientation is 36% in R82A and 84% in the WT. Under these conditions one may expect that the initial rate in R82A vesicles would be equal to 36/84 = 0.43 that of the WT, if the quantum yield of the proton transfer is the same. The experimentally observed value of 0.41 is within the limits of accuracy of this estimate. Thus, both flash and continuous light measurements in the vesicles indicate that the quantum yield of proton transfer in R82A is close to that in the WT. However, the kinetics of

the proton release is drastically delayed.

DISCUSSION

Electrostatic Model To Account for the Features of the R82A Mutant. The R82A mutant shows several spectral and photochemical features which are different from those in the WT. (1) The pK of the purple-to-blue transition shifts from 2.6 to 7.2 in R82A, similar to the shift described for the mutant expressed in *E. coli*. (2) The pH dependence of dark adaptation changes drastically. (3) The rise time to M formation is about 2 orders of magnitude faster in R82A than in the WT (at neutral pH) and is pH independent; the activation energy of M formation is decreased. (4) The rate of proton release is greatly slowed in the mutant.

Arginine is presumably a charged residue in bR, bearing a positive charge on its guanidinium group. Substitution of Arg82 by a neutral alanine residue should drastically change the electrostatic interactions in the vicinity of this residue. Thus, it is interesting to try to interpret some of these features on the basis of changes in the energy of electrostatic interactions. Below are some estimations and models based on this approach.

pK of the Purple-to-Blue Transition and the Dielectric Distance between Arg82 and Asp85. The pH titration curve to the purple-to-blue transition in R82A indicates that only one directly involved residue deprotonates during this transition (between pH 6 and pH 9). Metz et al. (1992) presented arguments indicating that the purple-to-blue transition involves protonation of a single amino acid, Asp85. The shift in the pK of the purple-to-blue transition from 2.6 in the wild type to 7.2 in the mutant thus reflects the shift in the pK of Asp85. Qualitatively, it is reasonable that the positive charge on the Arg, if it is close to Asp85, decreases the pK of Asp85 by pushing protons away from $Asp85^-$. From a simple equation [see Bloomfield and Carpenter (1993)]

$$\Delta\psi = 2.3(RT/F)\Delta pK \quad (3)$$

the electrostatic potential, $\Delta\psi$, which causes the shift in the pK of Asp85, can be estimated. In our case $\Delta pK = 4.6$, and so $\Delta\psi = 4.6 \times 59$ mV $= 270$ mV. Using the point-charge approximation for the $\Delta\psi$

$$\Delta\psi(\text{mV}) = q/4\pi\epsilon\epsilon_0 r = 14400/r\epsilon \quad (r \text{ in Å}) \quad (4)$$

one can estimate the product of the distance between D85 and R82, r, and the effective dielectric constant, ϵ:

$$\epsilon r = 53 \quad (5)$$

Arg82 is located below Asp85 in the extracellular channel (Henderson et al., 1990). However, the exact position of a side chain of Arg82 has not been determined. It was suggested that it may be near Asp85, or downward away from Asp85 (closer to the membrane surface), or could move between the two positions. The positions of Asp85 and Arg82 relative to the extracellular aqueous boundary were determined using site-directed spin labels (Greenhalgh et al., 1991). They were found to be within 9 and 5 Å of the aqueous medium (in bR). Calculations of Bashford and Gerwert (1992) suggest a position of the Arg82 side chain closer to the Schiff base (rather than to the exterior of the membrane).

If we take the distance between Arg82 and Asp85 to be about 3–4 Å, then the effective dielectric constant must be about 18–13, which seems a reasonable value for the interior of a relatively hydrophilic extracellular channel.

On the Increase in the Rate Constant of the L to M Transition in the R82A Mutant. The lifetime of the L to M transition in the WT is about 85 μs at neutral pH, and in R82A it is approximately 1 μs. Assuming that this difference in rates is controlled just by the electrostatic potential

$$k = k_0 \exp(-\Delta\psi F/RT) = k_0 \exp(-\Delta\psi(\text{mV})/25) \quad (6)$$

(taking $k_0/k = 85$), one can estimate that $\Delta\psi$ between the Schiff base and Asp85 should be about 110 mv. This is consistent with the distance between the Schiff base and Arg82 being 7.0 Å and that between Asp85 and Arg82 being 4 Å (because, assuming a dielectric constant equal to 14, the electrostatic potential difference between the Schiff base and Asp85 produced by the positive charge on Arg82 would be 110 mV). This potential in the WT slows down the transfer of protons from the Schiff base to Asp85. Removal of this charge in the mutant allows the increase in the rate of the L to M transition.

Thus, both the pK change in R82A and the increase in the rate of the L to M transition can be explained in terms of electrostatic interactions between Arg82, Asp85, and the Schiff base if the latter two are located at a distance of about 4 and 7 Å from the Arg82 and the effective dielectric constant around the Schiff base and Asp85 is about 14. These estimations are approximate because they are based on a point-charge approximation and do not consider back-reactions and possible motions of other charges during protonation of Asp85. Nevertheless, they demonstrate that to a first approximation consideration of electrostatic interactions in the "Schiff base–counterion" complex can explain the observed features of the R82A mutant. The effect of the R82A mutation on M rise demonstrates the possibility of a strong electrostatic control of light-induced Schiff base deprotonation in bR. The fact that the electrostatic model provides a reasonable explanation for the shift in the pK of Asp85 and change in the kinetics of M formation indicates that Arg82 in the ground state is present as a charged residue.

In the WT the rise time of M formation increases from 85 to 6 μs as the pH is raised from 7 to 10. This increase probably is caused by the deprotonation of some amino acid residue in the vicinity of the Schiff base (see below). That M formation is pH independent in the mutant can be interpreted as an indication that Arg82 is the residue which gives this pH dependence to bR. This suggests that Arg82 deprotonates in the dark with a pK around 9. Another possibility would be the pH-dependent movement of positively charged Arg82 toward the outer surface of the purple membrane, which apparently becomes more negative at alkaline pH. We estimated that increasing the distance between the Schiff base and the Arg by about 3 Å (from 7 to 10 Å) would result in an increase in the rise time of M formation from 85 to 6 μs (assuming the dielectric constant being equal to 14). The motion of Arg82 during the photochemical cycle was proposed earlier (Braiman et al., 1988; Rothschild, 1992).

Changes in both the pK_a of the purple-to-blue transition and the kinetics of M formation are observed in several other mutants in which the net charge of the protein was not altered. An interesting exmaple is the D85E mutant, in which Asp85 was replaced by Glu (Lanyi et al., 1992). The rate of M formation in this mutant is highly accelerated. Glutamic acid has an extra $-CH_2-$ group in the side chain compared to the aspartate. In the framework of the electrostatic approach the effect of D85E mutation can be explained qualitatively in terms of a decrease in the distance between the negative charge on the carboxyl group and positively charged Schiff base, which would result in the acceleration of M formation.

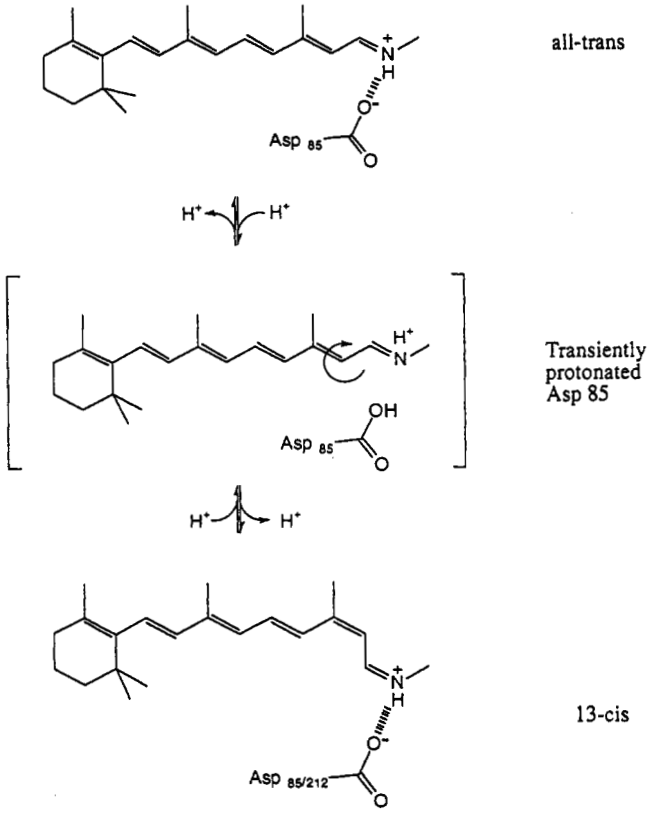


FIGURE 15: Scheme of thermal trans–13-cis isomerization in bR, suggesting an intermediate state in which Asp85 is transiently protonated. The (|||) indicates electrostatic interaction between Asp85 and the Schiff base.

pH Dependence of Dark Adaptation. Evidence for Involvement of the Transient Protonation of Asp85 in the Thermal Isomerization of the Chromophore in bR. The data in Figure 8 show an excellent correlation between the rate constant of dark adaptation and the fraction of blue membrane (or protonated Asp85) in R82A calculated from the Henderson–Hasselbach equation using the pK of Asp85 equal to 7.2. From this, it follows that the rate constant of dark adaptation, k_{da} (which is a sum of the rate constants of all-trans to 13-cis and 13-cis to all-trans thermal isomerization), as a function of pH is simply a product of a constant, k^0_{da}, and the fraction of protonated Asp85, f_{DH} (see eqs 1 and 2). This result suggests that protonation of Asp85 decreases the barrier for thermal isomerization (at least by 4.5 kcal/mol) and thus catalyzes the process. The simple equations (1 and 2) describe, over more than 3 orders of magnitude, the change in the rate of dark adaptation with pH. This indicates that the rate constant of isomerization in the purple state of the pigment, k^p_{da}, in which Asp85 is deprotonated, is at least 2 $\times$ 10^3 less than that in the blue state, k^b_{da}, and that dark adaptation in the mutant at pH 5–10 proceeds exclusively through the intermediate state, which is a blue form of the pigment (Figure 15). This blue species is transiently formed due to spontaneous protonation of Asp85.

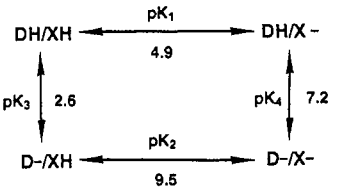
The catalytic effect of protonation of Asp85 on thermal isomerization of the chromophore of bR is likely to be important not only in the process of dark adaptation but also in the bR photocycle. Reisomerization of the chromophore from the 13-cis to all-trans configuration occurs during the transition from the N intermediate into the O intermediate. In both states Asp85 is protonated (Rothschild, 1992).

Our conclusion that protonation of Asp85 catalyzes thermal isomerization in bR is in agreement with the analysis of Warshel and Ottolenghi (1979), where it is proposed that negative charge near the Schiff base nitrogen will decrease the rate of thermal isomerization. This effect is explained by calculations of Warshel and Deakyne (1978), which indicated that twisting a double bond is associated with a shift of a positive charge toward the ring end. This implies that the negative charge near the ring will promote thermal isomerization while the negative charge near the Schiff base will decrease the rate of isomerization. In agreement with this is our conclusion that Asp85$^-$, a negative charge near the Schiff base nitrogen, greatly slows down thermal isomerization. Several studies (Orlandi & Schulten, 1979, Sheves & Baasov, 1984; Tavan et al., 1985; Seltzer, 1990; Milder, 1991; and references cited therein) pointed out that external charges affect delocalization of π-electrons in the chromophore which in turn strongly affect the rate of thermal isomerization. Asp85$^-$ stabilizes the positive charge on the Schiff base nitrogen and thus decreases the π-electron delocalization in the chromophore and increases the order of double bonds. Protonation of Asp eliminates this interaction. This causes an increase in π-electron delocalization and a decrease in the order of double bonds (C=N and 13C=14C), which results in a smaller barrier for isomerization. Along with this pure electrostatic effect, which probably plays the major role, one cannot exclude that Asp85 in its deprotonated form is involved in some network of hydrogen bonding with the Schiff base which poses additional restrictions on the thermal isomerization rates.

A Model for the pH Dependence of the Rate of Dark Adaptation in the Wild Type. Does the simple correlation between the rate of isomerization and the fraction of protonated Asp85 found for the mutant (eq 1) also hold for the wild type? The pH dependence of the rate of dark adaptation in the WT at 30 °C was measured by Ohno et al. (1977) and analyzed in a pioneering work of Warshel and Ottolenghi (1979). Our data for the WT taken at 20 °C and 150 mM KCl (Figure 8) are in general agreement with the data of Ohno et al. (1977). Between pH 3 and pH 5 a sharp decrease of about 2 orders of magnitude was found in the rate of dark adaptation. It remains almost unchanged between pH 5.5 and pH 9, while at higher pH (between 9 and 10) an additional decrease is observed. Warshel and Ottolenghi (1979) point out that the spectral changes and kinetics of dark adaptation of bR over a wide pH range (from 0 to 10) can be explained in terms of electrostatic interaction of the chromophore and three amino acids, A_1, A_2, and A_3, which deprotonate with pKs 0.8 2.8, and $\geq$8.5. The rate constant of thermal isomerization is the largest when A_2 is fully protonated. The rate constant for this state is k_2. The decrease of the rate between pH 3 and pH 5.5 is explained by the decrease in the concentration of protonated A_2 (which now can be identified as Asp85). The plateau corresponds to the rate constant of isomerization (k_3) in the state where A_1 and A_2 (Asp85) are deprotonated and A_3 is protonated. At pH >8 a further drop is associated with the deprotonation of a third amino acid, A_3, and a decrease in the rate constant of isomerization to k_4. The best fit was obtained with the constants k_2, k_3, and k_4 equal to 0.12, 5 $\times$ 10^{-4}, and $<5 \times 10^{-5}$, respectively. The ratio for the rate constants of isomerization in the blue state (k^b) versus purple state (k^p) is $k^b/k^p = k_2/k_3 = 0.24 \times 10^3$.

The finding that the rate of dark adaptation in R82A can be modeled over 3 orders of magnitude using the simple relationship (eq 1) stimulated us to develop a model for the WT based on the assumption that the fraction of blue membrane (or protonated Asp85) is the main parameter which determines the pH dependence of the rate of dark adaptation

according to eq 1. The model should account for the existence of electrostatic interactions not only between charged amino acids and the chromophore but also between amino acids themselves. It should directly incorporate the fact that loss of a positive charge in the vicinity of the Schiff base causes a large shift in the pK of Asp85, which changes the fraction of protonated Asp85 and the rate of dark adaptation. In further analysis we will use the notation D for Asp85 and X for another amino acid which deprotonates with a pK around 9–9.5 and changes the pK of Asp85 from 2.6 to 7.2. The two interacting groups, D85 and X, can exist in four different interconverting states:



Here pK_1 is the pK of X when D85 is protonated, pK_2 is the pK of X when D85 is deprotonated, pK_3 is the pK of D85 when X is protonated, and pK_4 is the pK of D85 when X is deprotonated. One of the consequences of this model is that the sum of pKs on the way from DH/XH to D^-/X^- does not depend on a particular pathway; pK_1 + pK_4 = pK_3 + pK_2. Thus the unknown pK_1 can be determined if the other three pKs are known.

We suggest that, in the states where Asp85 is deprotonated, the rate of thermal isomerization is at least 3 orders of magnitude less than in the states where Asp85 is protonated. The fraction of protonated Asp85 as a function of pH is described by the following equation, which can be easily obtained from consideration of ratios of different states in the equilibrium:

$$f_{DH}(pH) = \alpha/(\alpha + \beta\gamma) \quad (7)$$

where $\alpha = 1 + 10^{pH-pK_1}$, $\beta = 1 + 10^{pH-pK_2}$, and $\gamma = 10^{pH-pK_3}$. The f_{DH}(pH) calculated from this model for the case pK_1 = 4.9, pK_2 = 9.2, and pK_3 = 2.6 (and pK_4 = 7.2) is shown in Figure 8. The analytical curve obtained from eq 7 is a good approximation of the experimental points of the rate constant of dark adaptation in the WT. This demonstrates that in the WT the rate constant of dark adaptation (thermal isomerization) is directly related to the fraction of blue membrane (fraction of protonated Asp85) by a simple equation (1). Our model is a special case of the model of Warshel and Ottolenghi (1979). The main difference is that our model directly accounts for the changes in electrostatic interaction between D85 (A_2) and X (A_3) with pH. Some deviation between the theoretical curve and experimental points is seen at pH around 4. Apparently, this is because for simplicity we ignored the fact that the purple-to-blue transition in the WT is described by a pK plot with $n > 1$ (n = 1.5) and considered n = 1.

The identity of group X still has to be established. Many studies have characterized an alkaline form of bR which is distinct from bR at neutral pH. The pK of the transition between the two forms is around 9. Under alkaline conditions, the rate of formation of the M intermediate increases (Kalisky et al., 1981; Hanamoto et al., 1984; Liu, 1990); both the tryptophan and chromophore absorption bands shift (Balashov et al., 1991); and the rate of isomerization (Ohno et al., 1977) and the isomeric ratio of the chromophore in the dark-adapted state (Kaulen & Povstanogova, 1990) change. It was proposed that the group deprotonating with pK around 9 could be a tyrosine (apparently Y185 or Y57) (Hanamoto et al., 1984; Balashov et al., 1991) or a lysine (Maeda et al., 1988). The pK of the guanidinium group of Arg in solution is very high (about 12), and this seems to rule out the possibility that it can deprotonate with a pK of 9.5 in bR. However, if there is a positive charge near it (a cation or another positively charged residue), the pK of Arg82 could be substantially reduced. Another possibility is a water molecule, which may be associated with Arg82 (Braiman et al., 1988) and have a lower pK of dissociation. Finally, the third possibility is the motion of Arg82 toward the outer surface at high pH. One can suggest that Arg82 does not deprotonate at pH 9–10 but just moves away from Asp85 toward to the membrane boundary. This also would explain the pH dependence of dark adaptation and increased rate of M formation at high pH.

In the above discussion we have focused on the role of Asp85 and Arg82 (or group X) in controlling the rate of thermal isomerization in bR. However, this does not exclude the possible contribution of other charged or ionizable residues located in the retinal binding pocket, like Asp212, Tyr57, and Tyr185, in also affecting the pK of Asp85 and the rate of isomerization. In this context it is interesting to note that the rate constant of dark adaptation in the WT at pH 1.5 when D85 is protonated is an order of magnitude faster than in R82A at pH 5.5 (where D85 is also fully protonated). This suggests that besides Asp85 there are some other factors controlling the rate constant of thermal isomerization of the bR chromophore.

On the Possible Role of Arg82 in Proton Release. In the absence of R82 the release of a proton from the membrane [from the proton release group X in the designation of Zimányi et al. (1992) or Ebrey (1993)] is altered drastically. In the mutant it occurs after reprotonation of the Schiff base, apparently directly from Asp85 or some other group. One could conceive of two different ways in which Arg82 may be involved in proton release: (1) direct and (2) indirect, or coupled. (1) Since the positive charge on Arg82 influences the pK of Asp85, then there should also be the opposite effect: upon protonation of Asp85 during M formation, the pK of Arg82 should decrease. If one assumes that the pK ofArg82 in the ground state is around 9–9.5 rather than 12–13, then it could deprotonate and act as a proton release group. From eq 3 and 4 we estimate that if the distances from Arg82 to Asp85 and the Schiff base are 4 and 7 Å, respectively, the transfer of a proton from the Schiff base to Asp85 should decrease the pK of Arg82 by 2 pH units. If, in fact, the distance between Arg82 and Asp85 is less than 4 Å, then the ΔpK would be more than 2 pH units. According to Zimányi et al. (1992) the pK of the proton release group in the M intermediate is around 6. This suggests that under some conditions (pK 9–9.5 in the ground state and close location to Asp85) Arg82 could act as a proton release group. (2) An alternative model for the involvement of Arg82 in proton release is that Arg82 controls, through electrostatic interaction, the pK of some other group which is directly involved in the proton release. Evidently, further experiments are needed to elucidate the ground state pK of Arg82, its change during the photocycle, and the mechanism of involvement of Arg82 in the process of proton release.

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