Guanidinium Restores the Chromophore but Not Rapid Proton Release in Bacteriorhodopsin Mutant R82Q

Robert Renthal,*# Yong-Ji Chung,* Ricardo Escamilla,* Leonid S. Brown,§ and Janos K. Lanyi§
*Division of Earth and Physical Sciences, University of Texas at San Antonio, San Antonio, Texas 78249; #Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284; and §Department of Physiology and Biophysics, University of California Irvine, Irvine, California 92717 USA

ABSTRACT Replacement of the Arg residue at position 82 in bacteriorhodopsin by Gln or Ala was previously shown to slow the rate of proton release and raise the pK of Asp 85, indicating that R82 is involved both in the proton release reaction and in stabilizing the purple form of the chromophore. We now find that guanidinium chloride lowers the pK of D85, as monitored by the shift of the 587-nm absorbance maximum to 570 nm (blue to purple transition) and increased yield of photointermediate M. The absorbance shift follows a simple binding curve, with an apparent dissociation constant of 20 mM. When membrane surface charge is taken into account, an intrinsic dissociation constant of 0.3 M fits the data over a range of 0.2–1.0 M cation concentration (Na^+ plus guanidinium) and pH 5.4–6.7. A chloride counterion is not involved in the observed spectral changes, as chloride up to 0.2 M has little effect on the R82Q chromophore at pH 6, whereas guanidinium sulfate has a similar effect to guanidinium chloride. Furthermore, guanidinium does not affect the chromophore of the double mutant R82Q/D85N. Taken together, these observations suggest that guanidinium binds to a specific site near D85 and restores the purple chromophore. Surprisingly, guanidinium does not restore rapid proton release in the photocycle of R82Q. This result suggests either that guanidinium dissociates during the pump cycle or that it binds with a different hydrogen-bonding geometry than the Arg side chain of the wild type.

INTRODUCTION

Light absorption by the protonated Schiff base of all-*trans* retinal initiates a series of proton transfer reactions that result in proton transport by bacteriorhodopsin (bR), the proton pump of the purple membrane of *Halobacterium salinarium* (Lanyi, 1995). Within ~100 μs of light absorption, the Schiff base transfers a proton to Asp 85, and at approximately the same time, a proton is released from the extracellular side of the membrane (Drachev et al., 1984; Varo and Lanyi, 1990). However, this proton comes from Glu 204 (Brown et al., 1995), whereas Asp 85 remains protonated through the M (Engelhard et al., 1985), N (Braiman et al., 1991), and O (Bousche et al., 1992) states of the photocycle. The connection between proton release by the Schiff base and proton release by Glu 204 remains to be determined. Direct interaction is not likely, as the groups are separated by more than 1 nm. Also, there is a delay between protonation of Asp 85 and dissociation of the carboxyl group at position 204 (Kandori et al., 1997). Speculation has centered on a possible role of Arg 82 (Henderson et al., 1990; Balashov et al., 1993; Brown et al., 1993; Brown et al., 1995; Scharnagl et al., 1995; Renthal et al., 1995), which is located between Asp 85 and Glu 204 in the three-dimensional structure of bR (Henderson et al., 1990). Replacement of the guanidinium side chain of Arg 82 by Gln (R82Q) or Ala (R82A) drastically slows the proton release kinetics, with release occurring directly from Asp 85 late in the pump cycle (Otto et al., 1990; Balashov et al., 1993; Brown et al., 1995). Titrations of Asp 85 have provided evidence for interaction between Arg 82 and Asp 85; replacement of Arg at position 82 by Gln or Ala raises the pK of Asp 85 ~5 pH units (Brown et al., 1993), and replacement by Lys raises the pK of Asp 85 ~1 pH unit (Balashov et al., 1995).

Several different roles for Arg 82 in the proton release reaction have been suggested. Initially, Arg 82 itself was proposed as the proton release site of the pump (Mathies et al., 1991), but this idea is inconsistent with recent pK estimates (Bashford and Gerwert, 1992; Brown et al., 1993; Sampogna and Honig, 1994). Another early suggestion was that the release site is a water molecule near Arg 82 (Braiman et al., 1988). More recently, it was proposed that proton transfer from the Schiff base to Asp 85 disrupts the ion pair between Arg 82 and Asp 85, changing the polarization of a chain of hydrogen-bonded water molecules, resulting in proton release from a surface amino acid at the end of the chain (Humphrey et al., 1994; Brown et al., 1995). Alternatively, there have been suggestions of a conformational change of Arg 82 between two positions, one near the Schiff base site and a second near the proton release site at the extracellular surface (Henderson et al., 1990; Bashford and Gerwert, 1992; Sampogna and Honig, 1994). Although Henderson et al. (1990) were unable to observe any electron density for Arg 82, there is enough room in the proton release channel to build a model of the side chain of Arg 82 in either position.

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Address reprint requests to Dr. Robert Renthal, Division of Earth and Physical Sciences, University of Texas at San Antonio, San Antonio, TX 78249. Tel.: 210-458-5452; Fax: 210-458-4469; E-mail: rrenthal@lonestar.utsa.edu.

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One way of testing the idea of a conformational change of Arg 82 is to remove the tether between the guanidinium functional group and the polypeptide backbone. In this paper, we report results of adding guanidinium and various derivatives to the R82Q mutant of bR, which lacks the guanidinium side chain. If the side chain is induced to move by backbone motions, then free guanidinium would fail to restore rapid proton release, as it is not connected to the backbone. However, if the guanidinium side chain has only a single binding site in the protein structure, then it should restore function, by analogy with the restoration of the chloride pump of halorhodopsin by addition of guanidinium to the R108Q mutant (Rudiger et al., 1995).

A preliminary account of this work was published as an abstract (Renthal and Chung, 1996).

MATERIALS AND METHODS

Materials

R82Q and R82Q/D85N *Halobacterium salinarium* were grown as previously described (Ni et al., 1990; Brown et al., 1993) and membranes were purified by the method of Oesterhelt and Stoeckenius (1974). Guanidinium derivatives and related compounds were obtained from the following sources: guanidinium chloride and ethylene diamine dihydrochloride from Sigma Chemical Co. (St. Louis, MO) and guanidinium sulfate, *N*-methyl guanidinium hydrochloride, *N*-ethyl guanidinium hydrochloride, 1,3-diaminoguanidinium monohydrochloide, and 1,4-diaminobutane from Aldrich Chemical Co. (Milwaukee, WI). Aminoguanidinium chloride was prepared from aminoguanidine bicarbonate (Aldrich) by addition of HCl.

Absorbance spectra

Typical samples had final concentrations of 10 μM bR and 20 mM sodium phosphate, pH 6.0. Varying concentrations of guanidinium or related compounds were added from stock solutions that were adjusted to the desired final pH, if necessary. Spectra were measured on an Aviv/Cary 14 instrument (Aviv Associates, Lakewood, NJ). Purple membrane samples were either suspended in buffer or polymerized in polyacrylamide gels (Mowery et al., 1979). The pH dependence of the purple-to-blue transition was measured on hydrated films of purple membrane that had been dried on quartz windows (Renthal and Regalado, 1991) or on polyacrylamide gels containing purple membrane.

Photocycle and proton transfer kinetics

Photocycle kinetics and rates of proton release and uptake were measured at single wavelengths, as previously described (Brown et al., 1995).

RESULTS

Effect of guanidinium and other bases on the absorbance spectrum of R82Q bR

Addition of guanidinium chloride to the blue form of R82Q bR caused a progressive shift in the absorbance maximum from 587 nm to lower wavelengths. Above ~0.3 M guanidinium chloride, the absorbance maximum appeared at 565 nm. Compared with the large absorbance shift observed with 0.2 M guanidinium chloride, almost no shift was observed with 0.2 M sodium chloride. Guanidinium sulfate showed a similar shift to guanidinium chloride (Fig. 1 and Table 1). Thus, the absorbance shift is due to guanidinium, not chloride.

The absorbance at 640 nm (A_{640}) was assumed to be related to the fraction of the blue form of R82Q (F_b) as follows:

$$F_b = (A_{640} - A_p)/(A_b - A_p)$$

where A_p is the absorbance at 640 nm of the purple form of R82Q well above pH 8, and A_b is the absorbance at 640 nm of the blue form of R82Q at pH 5. A graph of F_b versus guanidinium concentration gives apparent binding curves (Fig. 2). The data at pH 6.2 and 6.7 (Fig. 2 *A*) may be fit with an apparent dissociation constant of 0.02 M. Methyl- and ethyl-guanidinium chloride, as well as amino- and 1,3-diamino-guanidinium gave absorbance shifts similar to guanidinium chloride. Only small shifts were observed with putrescine (1,4-diaminobutane) or ethylene diamine (Table 1). Thus, the hydrogen-bonding geometry of the guanidinium group appears to be important in the interaction with bR. Furthermore, the absorbance shift can be attributed to the dissociation of D85, as the double mutant D85N/R82Q shows no guanidinium effect (Table 1).

Dependence of guanidinium binding on pH

The effect of 0.2 M guanidinium on the R82Q bR spectrum was measured as a function of pH (Fig. 3). The results show that the blue form of R82Q appears with an apparent pK of 6.3. The purple-to-blue transition reflects the dissociation of D85 (Subramaniam et al., 1990). The pK of D85 was measured as 7.1 for R82Q (Fig. 3 and Balashov et al., 1993; Brown et al., 1993), compared with ~3 for wild-type bR (Mowery et al., 1979; Fischer and Oesterhelt, 1979). Thus, guanidinium restores the purple chromophore of bR by lowering the pK of D85.

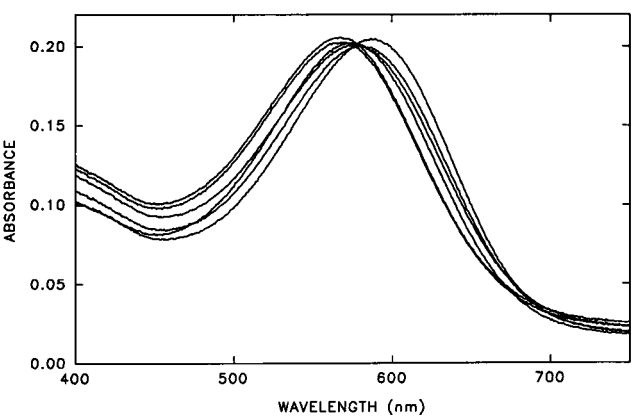

FIGURE 1 Addition of guanidinium to R82Q bR. Concentrations of guanidinium, from highest to lowest absorbance at 640 nm were 0 (pH 5.0), 0 (pH 6.2), 0.02, 0.04, 0.15, and 0.2 M. R82Q bR was polymerized in 4-mm-thick 5% polyacrylamide gels, 0.02 M phosphate, pH 6.2 (except as noted), and total cation was 0.2 M (combined guanidinium and sodium; anion is sulfate).

TABLE 1 Effect of guanidinium and related compounds on visible absorbance maximum of R82Q bR

Reagent	Absorbance maximum, 0.2 M reagent*
R82Q bR	
None	587
Guanidinium Cl	570
Guanidinium sulfate	565
Aminoguanidinium Cl	564
1,3-diaminoguanidinium Cl	565
Methyl guanidinium Cl	572
Ethyl guanidinium Cl	576
Ethylene diamine	581
Putrescine	583
NaCl	585
R82Q/D85N bR	
None	581
Guanidinium Cl	582
ETC-modified R82Q bR	
None	582
Guanidinium Cl	569

*Measured in 0.02 M phosphate, pH 6.0.

A simple physical model for the lowering of the pK can be derived by postulating that guanidinium binds to bR when D85 is in the unprotonated form:

$$RH \overset{K_a}{\rightleftharpoons} R + H^+ \quad (1)$$

$$RGu \overset{K_g}{\rightleftharpoons} R + Gu \quad (2)$$

where RH is bR with D85 protonated, RGu is bR with D85 unprotonated and guanidinium bound, K_g is the guanidinium dissociation constant, and K_a is the acid dissociation constant of D85. These equilibria predict that binding of guanidinium and H^+ are linked. The fraction of bR in the purple form depends on both the hydrogen ion concentration $[H^+]$ and guanidinium concentration [Gu]:

$$F_p = \frac{[Gu]/K_g + 1}{[Gu]/K_g + [H^+]/K_a + 1} \quad (3)$$

Linkage between the equilibria may be tested by measuring guanidinium binding as a function of pH. Both lines in Fig. 2 *A* are calculated from Eq. 3 with $K_a = 7.1$ (from Fig. 3). One line is measured at pH 6.7 and the other at pH 6.2. Both lines are calculated with $K_g = 0.02$ M. The experimental data fit the lines well, indicating that the linkage of Eq. 3 is valid. A term for an equilibrium representing the binding of guanidinium to the blue form of bR was also added to Eq. 3:

$$RHGu \overset{K_{gh}}{\rightleftharpoons} RH + Gu$$

However, this equilibrium was found to be unnecessary to model the results, and K_{gh} tended to make the fit to the experimental data poorer. Titration of R82Q below pH 3 shows that the low pH purple form of bR occurs to approximately the same extent in 0.2 M guanidinium chloride or sodium chloride (data not shown). The low pH purple form of bR has been shown to be due to halide binding near the Schiff base (Fischer and Oesterhelt, 1979; Varo and Lanyi, 1989; Renthal et al., 1990). Thus, the side chain of R82 does not appear to be necessary for this halide binding site.

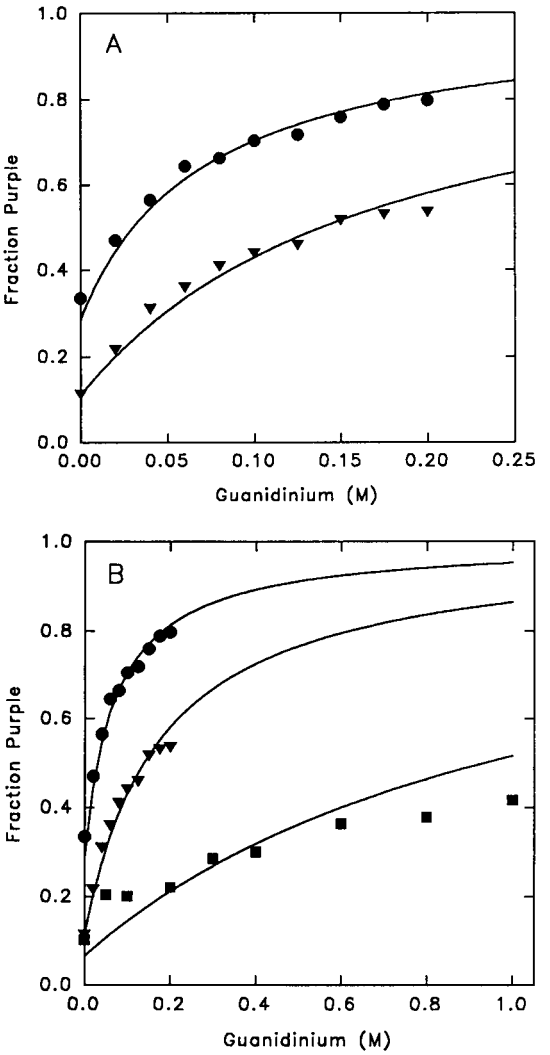

FIGURE 2 Binding of guanidinium to R82Q bR. Apparent guanidinium binding curves were obtained from data in Fig. 1 and additional measurements, assuming a simple equilibrium between guanidinium and a chromophore-linked site. (*A*) 0.2 M cation. ●, pH 6.7; ▲, pH 6.2. Both lines are calculated from Eq. 3 with guanidinium dissociation constant $K_g = 20$ mM. (*B*) ● and ▲, same as in *A*; ■, 1.0 M cation, pH 5.4. Lines calculated from Eq. 4 with $K_g' = 0.3$ M, $pK_a' = 5.9$, and surface charge density = -0.006 charges/Å^2. Cation is a mixture of Na^+ and guanidinium, and anion is sulfate.

Effect of surface charge

The proton equilibrium of Asp 85 by itself has been shown to be sensitive to the membrane surface potential (Szundi and Stoeckenius, 1989), which raises the possibility that guanidinium binding might similarly be affected. The concentration terms in Eq. 3 should actually be the surface

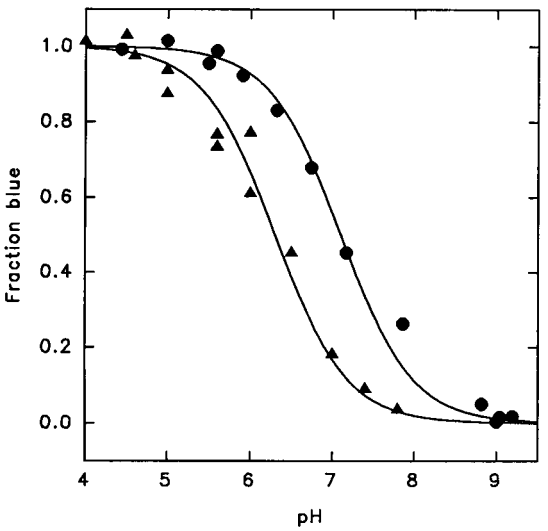

FIGURE 3 pH dependence of R82Q chromophore. Absorbance change was measured at 640 nm with varying pH. Purple membrane samples in polyacrylamide gels are similar to Figs. 1 and 2. ●, R82Q in 0.2 M Na^+; ▲, R82Q plus 0.2 M guanidinium. Buffers were as follows: pH 4.0–5.6, 0.02 M acetate; pH 6.0–7.8, 0.02 M phosphate. Lines fit with pK values of 6.3 and 7.1.

concentrations, rather than the bulk solution concentrations. Under the assumptions of the Gouy-Chapman equation, the concentration terms should be multiplied by exponential terms (i.e., $\exp(-\psi F/RT)$, where ψ is the surface potential at the membrane surface, F is Faraday's constant, R is the gas constant, and T is the absolute temperature):

$$F_p = \frac{[Gu]\exp(-\psi F/RT)/K_g' + 1}{[Gu]\exp(-\psi F/RT)/K_g' + [H^+]\exp(-\psi F/RT)/K_a' + 1} \quad (4)$$

where K_g' is the intrinsic dissociation constant for guanidinium and K_a' is the intrinsic acid dissociation constant for D85. Thus, the intrinsic equilibrium constants are

$$K_g' = K_g\exp(-\psi F/RT) \quad (5)$$

$$K_a' = K_a\exp(-\psi F/RT) \quad (6)$$

The value of ψ may be calculated from the surface charge density (McLaughlin, 1977). The pK of D85 shifts from 7.1 at 0.2 M Na^+ to 6.5 at 1.0 M Na^+. We assume the surface charge density does not change significantly over this pH range (i.e., we assume no phospholipid groups titrate). This is a reasonable assumption, as the surface pH is below the second pK of phosphate over this range. Using the Gouy equation, we calculated the surface potential from the surface charge density. The intrinsic pK of D85, pK_a', is related to the surface potential according to Eq. 5. We found that a surface charge density of approximately −6.5 charges per bR, or −0.006 charges per $Å^2$, on the extracellular membrane surface, gives $pK_a' = 5.9$ in Eq. 5 for both 0.2 M and 1.0 M Na^+. Thus, the exponential terms in Eqs. 5 and 6 are 14.6 and 4.3 for 0.2 M and 1.0 M cation, respectively (or ψ = −68 and −37 mV). Therefore, the intrinsic dissociation constant for guanidinium $K_g' = 0.3$ M. We measured the binding of guanidinium in 1.0 M cation at pH 5.4, and the data are shown in Fig. 2 *B* along with the 0.2 M cation data from Fig. 2 *A*. All three lines are calculated from Eq. 4 with $pK_a' = 5.9$ and $K_g'] = 0.3$. The only free parameter is the surface charge, which is −6.5 charges per bR for all three lines.

Effect of chemical modification of E74 on guanidinium binding

We previously found in wild-type bR that modification of E74 with a water-soluble carbodiimide (ETC) lowered the pK of D85 by ~1 unit (Renthal et al., 1995). This effect was interpreted as interference of the quaternary ammonium side chain of the carbodiimide with one of two possible positions of R82. In the R82Q mutant, ETC modification has no effect on the pK of D85 (data not shown). Furthermore, ETC does not inhibit guanidinium binding to R82Q (Table 1) although a steric effect by ETC limiting access to the guanidinium site might be expected. In addition, we saw a small effect (0.2 unit shift) of 1.0 M guanidinium on the pK of D85 of wild-type bR at low pH, where guanidinium might be expected to mimic the effect of ETC by binding near E204 and lowering the pK (data not shown).

Photocycle and proton release of R82Q in the presence of guanidinium

It was previously shown in the R82A mutant that the amplitude of photointermediate M is pH dependent, being proportional to the amount of the purple form of bR at a particular pH (Balashov et al., 1993). The M intermediate of R82Q rises faster than in the wild type and decays normally (Brown et al., 1995). At pH 6.0 in 2 M NaCl, where only ~10% of the maximal M formation occurs in R82Q, 0.2 M guanidinium restores approximately one-half of the maximal M (Fig. 4). Similar results were obtained in 0.2 M guanidinium without added salt. The pH dependence of M amplitude, which is a measure of the pK of Asp 85, follows the same trend as the results of direct titration (Fig. 3), indicating that the pK of Asp 85 is lowered by guanidinium added back to R82Q. However, surprisingly, transient pH changes induced by an actinic light pulse, measured by pyranine absorbance changes, show proton release occurs late in the photocycle, whether in the presence or absence of 0.2 M guanidinium (Fig. 5). The slow proton release in the presence of 0.2 M guanidinium was observed both with and without 2 M NaCl.

DISCUSSION

The removal by mutagenesis of the guanidinium side chain at position 82 in bR was previously shown to have drastic

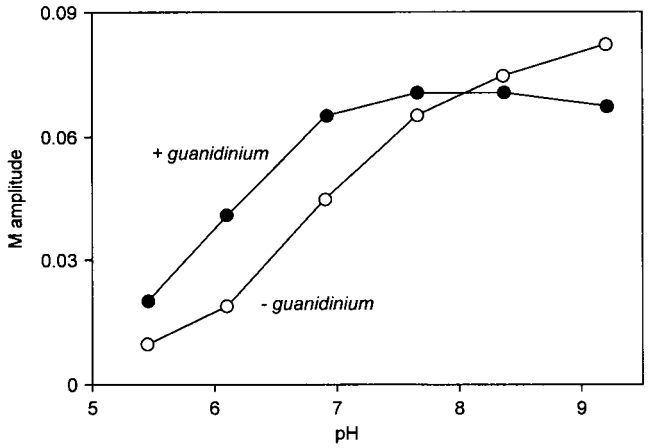

FIGURE 4 Amplitude of M as a function of pH. Absorption change at 410 nm was followed after flash illumination of R82Q at various pH values in the presence and absence of guanidinium chloride. The maximal amplitudes of these changes are plotted versus pH. Conditions were 2 M NaCl (○) or 1.8 M NaCl plus 0.2 M guanidinium chloride (●) and 0.1 M bis-tris-propane.

effects on bR. The pK of D85 is shifted to ~7 in R82Q, so that the pigment is blue at pH 6 (Subramaniam et al., 1990; Brown et al., 1993; Balashov et al., 1993). Although the purple form of R82Q undergoes a photocycle that includes the same intermediates as the wild type, the proton pump is altered. In R82Q, proton release occurs late in the pump cycle (Otto et al., 1990; Balashov et al., 1993; Brown et al., 1995). We now report that addition of guanidinium restores, in a concentration-dependent manner, the purple form of R82Q bR (Figs. 1–3). The guanidinium-induced purple form of R82Q cycles through the M photointermediate (Fig. 4). However, rapid proton release is not restored (Fig. 5). The guanidinium effect cannot be simply due to a decrease in surface acidity resulting from the lowering of the surface

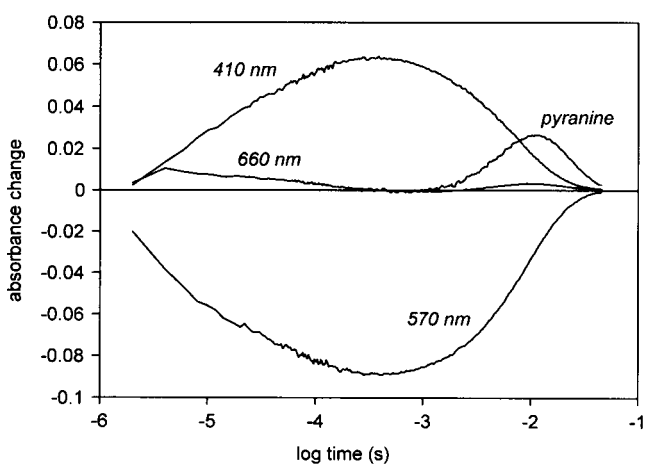

FIGURE 5 Photocycle and proton release in R82Q. Absorbance changes at 410, 570, and 660 nm were followed after flash illumination, and the net absorbance change of pyranine (trace without dye subtracted) at 457 nm. At 10 ms, the pyranine trace is the upper curve. Conditions were 2 M NaCl plus 0.2 M guanidinium chloride, pH 6.8, and 50 μM pyranine.

potential with increasing ionic strength, as an equal concentration of NaCl does not restore the chromophore (Table 1). Guanidinium is a powerful denaturing agent for proteins, and it is probably this aspect of its behavior that makes it useful for stabilizing photointermediates of bR (Yoshida et al., 1977; Pettei et al., 1977). However, the concentrations used for slowing the photocycle are typically in the range of 0.5–2 M, much higher than the amounts effective in the experiments reported here.

Our results are consistent with a discrete guanidinium binding site in R82Q. A likely location for this site would be near D85, similar to the side chain of R82 in the wild-type pigment. The absorbance changes resulting from increasing guanidinium concentrations, plotted as a binding curve (Fig. 2), fit a simple hyperbolic curve, indicative of a discrete, saturable binding site, with an apparent dissociation constant of 20 mM. We observe a shift of the pK of D85 in the presence of guanidinium (Fig. 3), which is most easily explained by formation of an ion pair between guanidinium and the carboxyl side chain of D85. The pK of D85 measured from a titration curve is related to the guanidinium concentration by Eq. 3: $K = K_a (1 + [Gu]/K_g)$. This suggests that an effective concentration of 250 M guanidinium would be required to shift the pK to the wild-type value of ~3. Effective concentrations of 10^2 to 10^5 M are typical of the entropic effect of tying down interacting groups in intermolecular interactions (Kirby, 1980).

Additional evidence for the specificity of binding was found by testing a variety of guanidinium analogs and organic amines (Table 1). All compounds tested that contained the guanidinium moiety restored the purple chromophore, but diamino alkanes had much smaller effects. The results in Table 1 suggest that the specific hydrogen-bonding arrangement of guanidinium is important in the interactions that lower the pK of D85. (The fact that a small effect is observed with putrescine and ethylene diamine is interesting in view of a recent modeling study of a diamino alkane binding site near D85 (Tan et al., 1996).) Govindjee et al. (1997) described effects of guanidinium on the pK of D85 in the mutant K129H. The pK shifts were smaller than we found with R82Q, and no dissociation constants were reported. It is not clear whether the guanidinium effects on R82Q and K129H (as well as the small effect on the wild type reported here and also by Govindjee et al.) are mediated through the same binding site.

If guanidinium binds to a specific site near D85 in the R82Q mutant, why is this not sufficient to restore rapid proton release? One possibility is that R82 is not involved in proton release. This view is taken by Alexiev et al. (1996), who have found a second mutation (G231C) near the carboxyl terminus that, in combination with R82A, restores rapid proton release. However, if R82 participates in a conformational change that occurs as part of the proton release mechanism, then the effect of the G231C mutation could be taken as evidence for the importance of a conformational change linked to proton release, rather than the lack of involvement of R82.

An alternative mechanism for proton release has been suggested as a change in polarization of a string of hydrogen-bonded water molecules in the proton release channel after proton transfer from the Schiff base neutralizes D85 (Brown et al., 1995). According to this idea, removal of the guanidinium at position 82 would disrupt the hydrogen-bonded chain linking D85 with the release site E204. Addition of guanidinium to R82Q should restore rapid proton release, if an intact H-bond network is sufficient for triggering proton release. As we do not observe rapid proton release in the presence of guanidinium, our results suggest that free guanidinium does not bind in an identical orientation to the arginine side chain of the wild type.

If a conformational change of R82 occurs after proton transfer to D85 from the Schiff base, then the guanidinium side chain must move away from its position near D85 early in the pump cycle. This suggests an explanation for the slow proton release in R82Q plus guanidinium. After protonation of D85, the interaction between guanidinium and its binding site is disrupted and the guanidinium dissociates from the membrane. Because it is not tethered to the polypeptide backbone, free guanidinium is unable to complete the proton release reaction. Thus, the fact that rapid proton release is not restored by guanidinium could be taken as evidence for the movement of the side chain of R82 during the proton release reaction.

Our results are in contrast to those of Rudiger et al. (1995), who have found that guanidinium restores full function to the halorhodopsin (hR) chloride pump in the R108Q mutant. This position is the analog of R82 in bR. However, a major difference between bR and hR is the direction of pumping. Chloride is pumped inward, and thus the situation is the reverse of the bR pump. From a structural standpoint, the Cl^- uptake step in hR is analogous to the interaction of R82 with D85. Thus, guanidinium binding actually occurs with the same protein state both in bR and hR.

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