2-1-1997

Mutation of a Surface Residue, Lysine-129, Reverses the Order of Proton Release and Uptake in Bacteriorhodopsin; Guanidine Hydrochloride Restores It

Rajni Govindjee
University of Illinois

Eleonora S. Imasheva
University of Illinois

Saurav Misra
University of Illinois

Sergei P. Balashov
University of Illinois

Thomas G. Ebrey
University of Illinois

See next page for additional authors

Mutation of a Surface Residue, Lysine-129, Reverses the Order of Proton Release and Uptake in Bacteriorhodopsin; Guanidine Hydrochloride Restores It

Rajni Govindjee,* Eleonora S. Imasheva,* Saurav Misra,* Sergei P. Balashov,* Thomas G. Ebrey,* Ning Chen,# Donald R. Menick,# and Rosalie K. Crouch#

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

ABSTRACT K129 is a residue located in the extracellular loop connecting transmembrane helices D and E of bacteriorhodopsin. Replacement of K129 with a histidine alters the pK_a's of two key residues in the proton transport pathway, D85, and the proton release group (probably E204); the resulting pigment has properties that differ markedly from the wild type. 1) In the unphotolyzed state of the K129H mutant, the pK_a of D85 is 5.1 ± 0.1 in 150 mM KCl (compared to ~2.6 in the wild-type bacteriorhodopsin), whereas the unphotolyzed-state pK_a of E204 decreases to 8.1 ± 0.1 (from ~9.5 in the wild-type pigment). 2) The pK_a of E204 in the M state is 7.0 ± 0.1 in K129H, compared to ~5.8 in the wild-type pigment. 3) As a result of the change in the pK_a of E204 in M, the order of light-induced proton release and uptake exhibits a dependence on pH in K129H differing from that of the wild type: at neutral pH and moderate salt concentrations (150 mM KCl), light-induced proton uptake precedes proton release, whereas it follows proton release at higher pH. This pumping behavior is similar to that seen in a related bacterial rhodopsin, archaerhodopsin-1, which has a histidine in the position analogous to K129. 4) At alkaline pH, a substantial fraction of all-*trans* K129H pigment (~30%) undergoes a conversion into a shorter wavelength species, P480, with $pK_a \approx 8.1$, close to the pK_a of E204. 5) Guanidine hydrochloride lowers the pK_a's of D85 and E204 in the ground state and the pK_a of E204 in the M intermediate, and restores the normal order of proton release before uptake at neutral pH. 6) In the K129H mutant the coupling between D85 and E204 is weaker than in wild-type bacteriorhodopsin. In the unphotolyzed pigment, the change in the pK_a's of either residue when the other changes its protonation state is only 1.5 units compared to 4.9 units in wild-type bacteriorhodopsin. In the M state of photolyzed K129H pigment, the corresponding change is 1 unit, compared to 3.7 units in the wild-type pigment. We suggest that K129 may be involved in stabilizing the hydrogen bonding network that couples E204 and D85. Substitution of K129 with a histidine residue causes structural changes that alter this coupling and affect the pK_a's of E204 and D85.

INTRODUCTION

Bacteriorhodopsin (bR) is a chromoprotein, present in the purple membrane of *Halobacterium salinarium*, that acts as a light-driven proton pump. bR contains a retinal moiety covalently attached to the apoprotein at K216, via a protonated Schiff base. Upon excitation with light, the retinal undergoes isomerization from the all-*trans* to the 13-*cis* configuration, which initiates a photocycle coupled with the transport of a proton from the inside to the outside of the cell (for reviews see Oesterhelt et al., 1992; Rothschild, 1992; Ebrey, 1993; Khorana, 1993; Lanyi, 1993). In the wild-type (WT) bR at neutral pH, light-induced proton release occurs on the submillisecond time scale (hereafter referred to as early proton release) and proton uptake in tens of milliseconds. However, at low pH this sequence is reversed; whereas proton uptake still occurs in tens of milliseconds, it precedes proton release, which occurs late in the photocycle (Dencher and Wilms, 1975; Zimányi et al., 1992). We refer to the proton release that follows uptake as late proton release.

The roles of some of the key residues involved in the proton transport in bR have been identified. D85 is the primary proton acceptor upon light-induced deprotonation of the Schiff base during the L → M transition, and D96 is the proton donor to the Schiff base during the M → N transition (see reviews above). It has recently been suggested that the residue that releases the proton into the extracellular medium upon the formation of the M state is E204 (Scharnagl et al., 1994, 1995; Brown et al., 1995; Richter et al., 1996; Govindjee et al., 1996; Sampogna and Honig, 1996). Several other residues in the retinal binding pocket (e.g., R82 and Y57) have been shown to affect proton release and/or uptake. Late proton release has been reported at neutral pH in R82A, R82Q (Otto et al., 1990; Balashov et al., 1993; Cao et al., 1993; Govindjee et al., 1996), and Y57F (Lanyi, 1993; Govindjee et al., 1995), ostensibly due to an elevated pK_a of E204 in M in these mutants.

Another residue that may be important in the proton transport process is K129, which is located on the extracellular loop connecting the transmembrane helices D and E (Grigorieff et al., 1996). There are conflicting reports in the literature about the consequences of modifying K129. Sev-

Received for publication 7 August 1996 and in final form 12 November 1996.

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

0006-3495/97/02/886/13 $2.00

eral studies report no unusual effects caused by the substitution of K129 with cysteine or other residues (Altenbach et al., 1990; Scherrer et al., 1992; Alexiev et al., 1994a,b). Heberle and Dencher (1990, 1992) and Alexiev et al. (1994a,b) have shown that bR functions normally when fluorescein is covalently attached to K129 or to C129. On the other hand, chemical modification of K129 with fluorescamine seems to abolish proton release in bR (Singh and Sonar, 1988), and acetylation of K129 (and other lysines) inhibits proton release but not proton uptake (Takeuchi et al., 1981). We have found that the order of light-induced proton release and uptake is ionic strength dependent in acetylated bR: at low to moderate salt concentration, proton release is delayed and occurs after proton uptake, but at high salt concentration normal proton release before uptake is observed (Q. G. Li et al., unpublished observations). A similar ionic strength dependence of proton release and uptake was reported for one of the archaerhodopsins, aR-1, which contains all of the major residues associated with the proton pumping process in bR, but has a histidine residue in the position analogous to K129 in bR (Lukashev et al., 1994).

We present data which suggest that when K129 is replaced with a histidine, the pK_a's of two of the key residues in the proton transport pathway, D85 and E204, are altered. The pK_a of D85 is raised and the pK_a of E204 is reduced in the unphotolyzed K129H pigment, compared to unphotolyzed wild-type bR. (Bacteriorhodopsin mutants are designated as K129H, etc., where the first letter and number represent the wild-type residue and the second letter represents the substituted residue.) The pK_a of E204 in the M state is greater in K129H than in wild-type bR. As a result of the higher pK_a of E204 in the M state, the order of proton release and uptake is reversed in K129H at neutral pH relative to the WT pigment. The guanidinium ion restores the normal order of proton release and uptake in K129H at neutral pH, probably by specifically lowering the pK_a of E204 in the M intermediate.

MATERIALS AND METHODS

Site-directed mutagenesis of bR, the transformation of the K129H mutant into *H. salinarium* strain IV-8, and the preparation of the purple membrane followed procedures described earlier (Balashov et al., 1993, 1995).

Flash-induced transient absorbance changes were measured with a home-built kinetic spectrophotometer (Govindjee et al., 1990). Actinic flashes at 532 nm were provided by a Nd:YAG laser, Quanta Ray DCR-11 (Spectra Physics, Mountain View, CA); flash intensity was adjusted to cause approximately 15–20% of the pigment to photocycle.

Light-induced proton release and uptake were measured with the pH indicator dye pyranine. The pK_a of pyranine is 6.7 and 7.2 in 1 M and 150 mM NaCl, respectively. The absorbance change of the dye was obtained by subtracting the kinetic traces at 460 nm in the presence and absence of the indicator as described earlier (Govindjee et al., 1996). Kinetic analysis of the dye absorbance traces was performed using the Kaleidagraph software package (Synergy Software, Reading, PA) and our own programs. Dye traces were fit as sums of either one or two negative exponentials (representing the early and late proton release phases when present) and one positive component (representing the proton uptake phase), with the added constraint that the sum of the positive and negative components be equal to zero (see Govindjee et al., 1996).

Dark adaptation measurements were carried out in 150 mM KCl on membranes immobilized in acrylamide gels, as described by Balashov et al. (1996). The gels were incubated overnight at the respective pH. Light adaptation was carried out by illumination with a 500-W projector and Corning CS 3-71 + 4-94 + 4-71 filters (λ = 430–550 nm) for 5 min (above pH 8 the time was reduced to ~2 min) at 20°C. The rate of dark adaptation was followed as a time-dependent decrease in absorbance at 580 nm. All measurements were carried out on an AVIV 14DS spectrophotometer (Aviv Associates, Lakewood, NJ).

RESULTS

The pK_a of D85 in the K129H mutant of bacteriorhodopsin

The pK_a of D85 in the K129H mutant was determined from the pH dependence of the rate constant of dark adaptation, as this rate constant is proportional to the fraction of protonated D85 in the unphotolyzed state (Balashov et al., 1993, 1995, 1996), and from the titration of the purple-to-blue membrane conversion of the pigment (Subramaniam et al., 1990; Metz et al., 1992).

pH dependence of the rate constant of dark adaptation

The rate constant of dark adaptation in K129H at neutral pH is an order of magnitude faster than in the WT. The pH dependence of the rate constant of dark adaptation has a complex titration curve with two transitions (Fig. 1 *A*), as in the wild-type bR. The complex titration curve has been associated with the coupling of the pK_a's of D85 and group X′ (Balashov et al., 1995). Strong evidence has been presented that X′ is E204 (Richter et al., 1996). In K129H, the lower transition, which is associated with the protonation of D85, has a pK_a of ~4.9 in 150 mM KCl. The second transition has a pK_a of 8.0, which can be attributed to E204. The two pK_a's differ considerably from the analogous pK_a's in the WT (2.6 and 9.7).

Absorption changes on acid titration

The pK_a of D85 was also determined by the titration of the purple-to-blue membrane conversion, which reflects the protonation of D85 (Subramaniam et al., 1990; Metz et al., 1992). Fig. 2, *A* and *B*, shows the absorption spectra of the K129H pigment at different pH values. At neutral pH the λ_{max} of the dark-adapted pigment is approximately 555 nm. Decreasing the pH from 7.0 to 4.3 causes an increase in absorbance at long wavelengths, and the λ_{max} of the pigment shifts to ~600 nm (at pH 4.3) because of the purple-to-blue membrane conversion (Fig. 2 *A*), in which D85 is protonated. A plot of the ΔA at 650 nm, representing the purple-to-blue membrane conversion, as a function of pH is shown in Fig. 2 *C*. The blue membrane can be traced up to pH 9 by measuring absorbance changes at 650 nm, where this species predominantly absorbs. A substantial amount of

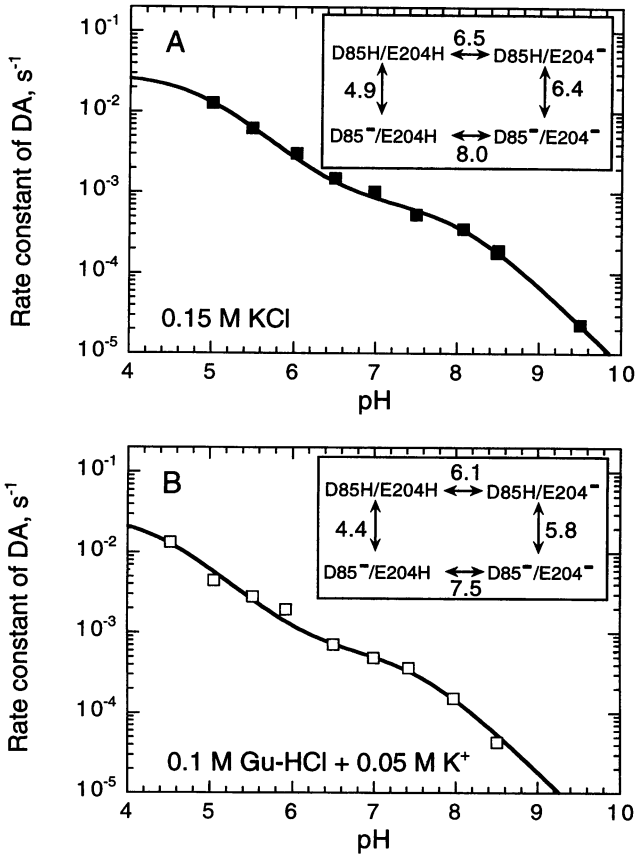

FIGURE 1 pH dependence of the rate constant of dark adaptation in K129H pigment at 20°C. (*A*) In 150 mM KCl. (*B*) In 150 mM guanidine hydrochloride. Solid lines represent fit of the data with the coupled protonation states model by Balashov et al. (1993, 1995, 1996). The pK_as of D85 and E204 determined from the fit are indicated in the insets.

blue membrane (about 6% of its maximum value) is present at pH 7. The plot of the data on a log scale shows that in addition to a main transition with a pK_a of ~5.1-5.2, there is another transition with $pK_a \approx 8.0$. The titration curve for the blue membrane in K129H mutant can be fitted with the model of two interacting residues (D85 and X′) (Balashov et al., 1995, 1996). It indicates that the pK_a of D85 is ~5.2 in K129H, and the pK_a of X′ (E204) is ~8.0. According to the fit, upon protonation of D85, the pK_a of E204 changes from 8.0 to 6.5, and the pK_a of D85 shifts from 5.2 to 6.7 upon deprotonation of E204. These values are close to those obtained from the fit of the pH dependence of the rate constant of dark adaptation (see Fig. 1 *A*). The fact that the pK_a of D85 determined from the dark-adaptation data is slightly lower (4.9 versus 5.2) may be due to the formation of an acid purple species in which the spectral effect of the protonation of D85 is offset by the binding of an anion like Cl^- near the Schiff base (Fischer and Oesterhelt, 1979; Mowery et al., 1979). Because the titrations were performed in the presence of Cl^- anions, the formation of a small amount of the acid purple species would cause an underestimation of the amount of protonated D85 at low pH in the spectroscopic titration measurements (because its absorbance is blue-shifted from that of the blue membrane). The acid purple species does not exhibit dark adaptation (Fischer and Oesterhelt, 1979; Mowery et al., 1979) and thus does not perturb the dark adaptation measurements. We used Cl^- in these experiments so that we could compare these results to similar experiments with guanidine hydrochloride (see below).

The pK_a of E204 in the unphotolyzed K129H pigment

The pK_a of the proton release group (presumably E204) in the unphotolyzed pigment was determined by measuring the pH dependence of 1) the rate constant of dark adaptation and titration of D85 (Balashov et al., 1993, 1995, 1996; Richter et al., 1996); 2) the formation of a shorter wavelength species, P480; and 3) the total amplitude of the M photointermediate (see Discussion).

pH dependence of the rate of dark adaptation and titration of D85

The transition at higher pH seen in the pH dependence of the rate constant of dark adaptation (Fig. 1 *A*) and titration of blue membrane (Fig. 2 *C*) is associated with the protonation state of group X′ (Balashov et al., 1993, 1995, 1996). X′ should be identified as E204, according to Richter et al. (1996), indicating that the pK_a of E204 is ~8.0 ± 0.1 in the unphotolyzed K129H pigment.

Formation of P480 (alkaline titration of the absorption spectrum)

On alkalinization from pH 7 to ~9, a dark-adapted membrane suspension of K129H pigment exhibits a decrease in extinction coefficient, and the λ_{max} of the pigment blue-shifts to ~545 nm (Fig. 2 *B*). With increasing pH, the pigment is partially transformed into a species absorbing at shorter wavelengths, which we term P480. Note that this shift appears to be distinct from the complete transformation into a blue-shifted species observed in all bacteriorhodopsin pigments at very high pH (>12) and attributed to the deprotonation of the Schiff base (Druckmann et al., 1982). Partial transformation of the normal purple species, present at neutral pH, into P480 has been observed in wild-type bR and in the Y57F and Y57N mutants (Balashov et al., 1991; Govindjee et al., 1992, 1995). Because 576 nm is the isosbestic point in the purple-to-blue membrane transition of K129H (see Fig. 2 *A*), the pH-dependent changes at 576 nm are due to P480. The pK_a of P480 formation was determined by plotting ΔA at 576 nm as a function of pH. As shown in Fig. 2 *D*, the pK_a of P480 formation in K129H is ~8.1 ± 0.1 in 150 mM KCl. The transformation of the purple pigment into P480 is only partial in the pH range examined, saturating above pH 9. The amount of P480 seen in K129H at pH 9–10 is ~3 times larger than in the WT at these pH values (Balashov et al., 1991).

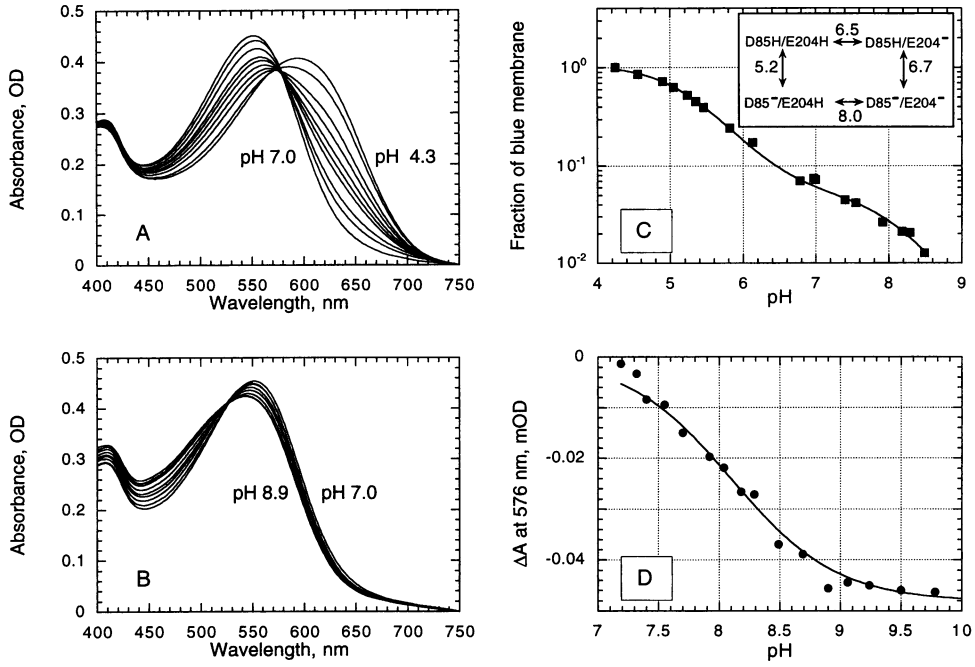

FIGURE 2 pH dependence of the absorption spectrum of dark-adapted K129H pigment in 150 mM KCl/25% glycerol at 20°C. (*A*) Absorption spectra taken at pH 4.3, 4.6, 4.9, 5.1, 5.2, 5.4, 5.5, 5.8, 6.1, 7.0, showing mainly the blue-to-purple transition. (*B*) Absorption spectra taken at pH 7.0, 7.2, 7.3, 7.4, 7.5, 7.7, 7.9, 8.0, 8.2, 8.3, 8.5, 8.7, 8.9, showing mainly the conversion of the pigment into P480. (*C*) Fraction of blue membrane as a function of pH. The experimental points were taken from *A* and *B* as a difference of absorbance at 650 nm between pH_i and pH 8.9, where the fraction of blue membrane is negligible (<0.01). The data were normalized to 1 and fitted with the coupled protonation states model (Balashov et al., 1995, 1996). The pK_a of the transitions given in the inset are derived from the fit. (*D*) pH dependence of formation of P480 as determined from the absorbance decrease at 576 nm (pH_i − pH 7.0 from *B*); the solid line shows that the transition has a $pK_a \approx 8.1$, $n = 1$. For clarity, *A* and *B* do not show all of the spectra corresponding to the points in *C* and *D*.

It is important to note that the pK_a of P480 formation in K129H coincides closely with the high pH transition in the pH dependence of the rate constant of dark adaptation (Fig. 1 *A*) and D85 titration (Fig. 2 *C*). As these transitions have been linked to the titration of E204, it is likely that the deprotonation of this residue is implicated in the partial transformation of the pigment into P480 or, inversely, that the partial transformation of the pigment into P480 seen in the WT (Balashov et al., 1991) and several mutants at pH 7–10 can serve as a useful diagnostic for the deprotonation of E204. In agreement with this possibility is the observation that in the E204Q mutant this partial transformation into P480 does not occur in the pH range between 7 and 11 (see Fig. 4 *B*). It occurs only at pH > 11.5, where the major transition of the pigment into P480 (or spectroscopically similar species) takes place in WT bR and mutants.

Illumination of a dark-adapted sample of K129H at pH 7.0 shows the typical changes associated with light adaptation in bR, namely a red shift in the λ_{max}, an increase in the extinction coefficient of the pigment, and the appearance of minor bands around 390 and 430 nm (the β bands characteristic of the all-*trans* pigment) (Fig. 3, *A* and *C*, *curve 1*). Illumination at pH 8.9 also results in a red shift of the pigment and an apparent decrease rather than an increase in the extinction coefficient, and shows a shoulder around 460–480 nm (Fig. 3 *B* and Fig. 3 *C*, *curve 2*). The difference spectra obtained by subtracting the dark-adapted from the light-adapted spectra at pH 7.0 and 8.9 are shown in Fig. 3 *C*. Note that the increase in absorbance at long wavelengths upon light adaptation at pH 8.9 is less than half of that observed at pH 7.0, whereas the minimum in the difference spectrum is red-shifted to 530 nm and is approximately two times larger compared to pH 7.0. In addition, there is an increase in absorbance at short wavelengths. The difference spectra suggest that illumination of K129H at pH 8.9 converts the 13-*cis* into all-*trans* pigment, and that the latter may be partly converted into P480 (see Discussion). Difference spectra between samples (light- and dark-adapted) at pH 8.9 and pH 7.0 also show that more P480 is formed with titration of the light-adapted pigment (Fig. 3 *D*).

pH dependence of the amplitude of the M photointermediate

The amplitude of the M intermediate formed from bR after a flash increases as the pH increases from 4 to 7, reflecting mostly the conversion of blue membrane to purple membrane, as only the latter can form M. Above pH 7 the M amplitude decreases, reaching a steady level around pH 10 (Fig. 4 *A*, *curve 1*). The percentage decrease in the M amplitude (~39%) is similar to the decrease in the absorbance of the pigment (~33%; see Fig. 3 *D*, *curve 2*). The

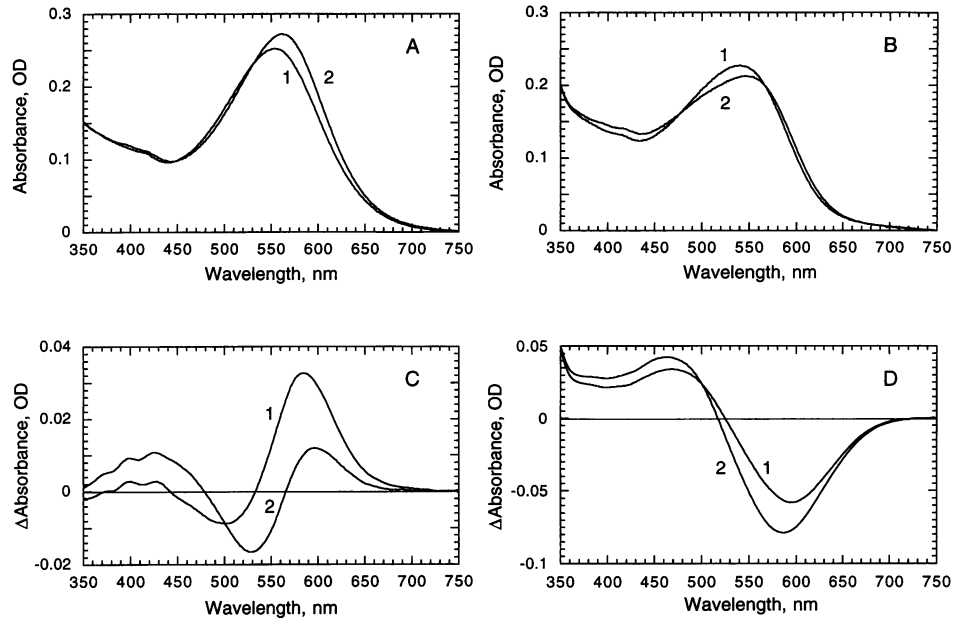

FIGURE 3 Changes of absorption spectra of K129H pigment upon light adaptation at pH 7.0 and 8.9. (*A*) Absorption spectra of dark-adapted (*curve 1*) and light-adapted (*curve 2*) K129H at pH 7.0. (*B*) Absorption spectra of dark-adapted (*curve 1*) and light-adapted (*curve 2*) K129H at pH 8.9. (*C*) Light-adapted minus dark-adapted difference spectra at pH 7.0 (*curve 1*) and pH 8.9 (*curve 2*). (*D*) Difference spectra pH 8.9 minus pH 7.0 of dark-adapted (*curve 1*) and light-adapted (*curve 2*) pigment.

pK_a of the decline in M amplitude is approximately 8.2 (in 150 mM KCl; pK_a ~7.3 in 1 M NaCl). This value is similar to the pK_a for the alkaline transformation of the pigment into P480 (Fig. 2 *D*) and the higher pK_a in the pH dependence of the rate constant of dark adaptation (Fig. 1 *A*) (summarized in Table 1). Thus the M amplitude in the K129H pigment increases upon the deprotonation of D85 and subsequently decreases at high pH because of the partial transformation of the pigment into P480 associated with the deprotonation of another amino acid residue, possibly E204.

To investigate whether the decrease in the amplitude of the M photointermediate at high pH is related to the deprotonation of E204, we measured the pH dependence of the M amplitude in the E204Q mutant. If the deprotonation of E204 is responsible for the decrease in the M amplitude, then there should be no pH dependence in the E204Q mutant. The M amplitude indeed remains essentially unchanged in the E204Q pigment in the pH range studied (pH 5.6–10.5; Fig. 4 *A*). The E204Q pigment also shows no P480 formation up to pH 11.5 (Fig. 4 *B*), providing support for the contention that deprotonation of E204 facilitates the formation of P480 with a pK_a of ~8 in K129H.

Several phenomena occurring in parallel are caused by the titration of E204

The combined evidence suggests that the titration of E204 in K129H is responsible for 1) the decline in M amplitude at high pH and 2) the partial transformation of the pigment into P480, which is not able to be photoconverted into the M photointermediate. Both of these transitions occur with the same pK_a as the alkaline transition in the pH dependence of the rate constant of dark adaptation, which has already been identified with the titration of E204 (Balashov et al., 1996; Richter et al., 1996). In addition, these transitions are not found in a mutant in which E204 has been substituted with an unprotonable residue, E204Q (Fig. 4, *A* and *B*). The deprotonation of E204 appears to facilitate partial transformation of the pigment to P480, but cannot be the sole determining factor, as the pigment is not completely transformed to P480 at pH 10. Both the formation of P480 and the decline in the amplitude of M (Fig. 4) saturate at high pH, suggesting that another process, possibly the titration of a second residue, is required for the complete transformation of the pigment.

Photocycle kinetics and the pK_a of E204 in the M state of K129H

Photocycle kinetics

Fig. 5 *A* shows the flash-induced absorbance changes, at neutral pH, of the photocycle intermediates M and O measured at 410 nm and 680 nm, respectively, and the absorbance decrease and recovery of the pigment measured at 580 nm. The lifetime of M formation is very fast (compared to wild-type bR), $\tau \approx 14$ μs, in 150 mM KCl. The decay of the M intermediate shows three components, $\tau_1 \approx 5$ ms (amplitude ~15% of the total signal), $\tau_2 \approx 17$ ms (~75% of the total signal), and $\tau_3 \approx 80$ ms (<10% of the signal). The lifetime of decay of the major component of M is similar to the lifetime of O decay, suggesting the presence of an M, N, and O equilibrium that decays during the recovery of the pigment, $\{M \leftrightarrow N \leftrightarrow O\} \rightarrow bR$. Fig. 5 *B* shows the light-induced pH changes as measured by the absorbance changes of the pH-sensitive dye pyranine and the O rise ($\tau \approx 10$ ms) and decay ($\tau \approx 17$ ms) kinetics at neutral pH. The rate of proton uptake roughly corresponds to the rise of the O intermediate, whereas the late proton release is slightly slower than the decay of O.

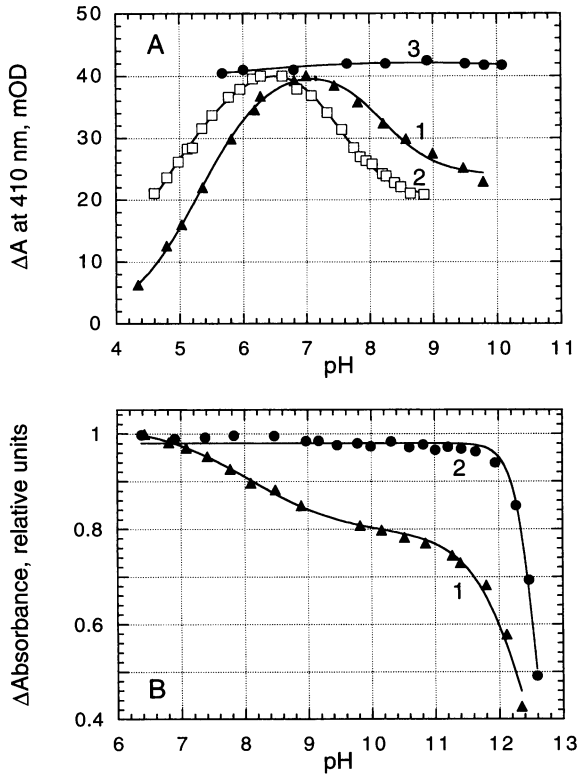

FIGURE 4 (*A*) pH dependence of the amplitude of the flash-induced absorbance change at 410 nm due to the M intermediate in K129H and E204Q. In K129H (*curves 1 and 2*), the M amplitude increases because of the blue-to-purple membrane conversion, and decreases because of the transformation of the purple pigment into the alkaline species (P480); in 150 mM KCl (*curve 1*) the respective pK_as are ~5.2 and 8.2; in 150 mM guanidine hydrochloride (*curve 2*) the respective pK_as are ~4.8 and 7.5. In the E204Q pigment (*curve 3*) the M amplitude is pH independent from pH 5.6 to 10. All three measurements have been normalized at pH 6.8. $\lambda_{actinic}$ = 532 nm, 20°C, sample OD at λ_{max} = 0.45. (*B*) pH dependence of transformation of purple membrane into the P480 species in the K129H (*curve 1*) and E204Q (*curve 2*) mutants. The absorbance of pigments at pH 6 was taken as 1. The absorption decrease due to the transformation into P480 was determined from the absorbance changes at 576 nm for K129H pigment (see Fig. 2 *D*) and at 565 nm for the E204Q pigment. Dark-adapted pigments were used.

The pK_a of E204 during the photocycle in the M state was determined by measuring the fraction of early proton release as a function of pH, as reported earlier for the wild-type and R82Q pigments (Zimányi et al., 1992; Govindjee et al., 1996). In wild-type bR, when the bulk pH is below the pK_a of the proton release group in the M state, the order of proton release and uptake is reversed (Dencher and Wilms, 1975; Zimányi et al., 1992). Because of the large proton back-pressure, E204 cannot deprotonate in M; a proton is released coincident with the O → bR transition (Govindjee et al., 1996). Thus the pK_a of the proton release group in the M state can be determined by measuring the pH dependence of the fraction of early and late proton release (Zimányi et al., 1992; Govindjee et al., 1996). Unlike wild-type bR, in the K129H pigment most of the proton uptake occurs before release. Only ~25-30% of proton release is observed before uptake near neutral pH. Fig. 6 shows the flash-induced absorbance changes of the pH indicator dye pyranine at different pH values. In 150 mM KCl, the fraction of early proton release increases from ~0.3 at pH 6.8 to ~0.9 at pH 7.9 (Fig. 6 *A*), with the $pK_a \approx 7.0 \pm 0.1$ (see Fig. 6 *A*, *inset*). Thus the pK_a of E204 in the M state is ~7 in 150 mM salt (~6.4 in 1 M salt; Fig. 6 *B*). (These estimates may be slightly altered if there is a kinetic phase of proton release with a time constant similar to that of proton uptake. Such a component would not be observable in our dye measurements, but cannot be excluded as discussed earlier (Balashov et al., 1995, 1996).)

Effects of guanidine hydrochloride on the pK_a's of D85 and E204 and on the order of proton release and uptake

Moderate concentrations (50–150 mM) of guanidine hydrochloride (Gu-HCl) have been shown to affect the pK_a of D85 in R82 mutants of bR (Alexiev et al., 1996; Renthal and Chung, 1996). In the R82A mutant, Gu-HCl restores the normal order of proton release and uptake, which is ordinarily reversed at neutral pH (Alexiev et al., 1996). Gu-HCl was also found to restore chloride pumping in a defective R108 mutant of halorhodopsin (Rüdiger et al., 1996). To check whether Gu-HCl functions (in bR) solely by substituting for the guanidinium groups of the R82, or can itself affect the pK_a of the proton release group, we studied its effect on the proton release process in the K129H mutant (in which R82 is present and ostensibly retains its function).

The pK_a's of D85 and E204 in the unphotolyzed pigment

Fig. 7 shows the pH dependence of the absorption spectrum of the K129H pigment in 150 mM Gu-HCl. Fig. 7 *A* shows the spectral changes in the pH range 6.6 to 4.0, reflecting the purple-to-blue membrane transition. The absorbance at 630 nm increases at low pH with a pK_a of ~4.6 ± 0.2 (Fig. 7 *C*, *curve 1*). Fig. 7 *B* shows the spectral changes from pH 6.6 to 9.7, depicting the alkaline transformation of the pigment into P480, and suggesting that the pK_a of E204 in the unphotolyzed pigment is ~7.7 ± 0.1 (Fig. 7 *D*).

As noted earlier, the unphotolyzed-state pK_a values of D85 and E204 can also be obtained from the pH dependence of the rate constant of dark adaptation of the pigment (Fig. 1 *B*). The pK_a's of the two transitions, reflecting the pK_a's of D85 and E204 in the unphotolyzed pigment, are ~4.4 and 7.5, respectively, in 150 mM Gu-HCl. The pK_a of D85 as determined from the rate of dark adaptation is slightly lower than the value from purple-to-blue membrane conversion (as was also seen in KCl, with no Gu-HCl present; see Table 1). As mentioned before, this may be due to the formation of the acid purple species.

Fig. 4 *A*, curve 2, shows the pH dependence of the M amplitude in 150 mM Gu-HCl. The M amplitude increases between pH 4.5 and 6.5 because of the blue-to-purple

TABLE 1 pK_a values of D85 and the proton release group, E204, in the K129H mutant determined by different methods

Amino acid residue	Process measured	pK_a in 150 mM KCl	pK_a in 150 mM Gu-HCl
D85 (in the unphotolyzed pigment)	Dark adaptation	4.9	4.4
	Purple-to-blue transition	5.2	4.6
	M amplitude	5.2	4.8
E204 (in the unphotolyzed pigment)	Dark adaptation	8.0	7.5
	Purple-to-blue transition	8.0	
	Formation of P480	8.1	7.7
	M amplitude	8.2	7.5
E204 (in the M intermediate)	Fraction of early proton release	7.0	<5.8*

*Approximation obtained from concentration dependence of Gu-HCl effect on early proton release.

membrane conversion, and then decreases above pH 6.5 and levels off near pH 8.5, reflecting the formation of P480 and the deprotonation of E204, the respective pK_a's being ~4.8 and 7.5.

Thus the three different measurements (summarized in Table 1) that reflect the protonation states of D85 and E204 suggest that the unphotolyzed-state pK_a's of both D85 and E204 are approximately 0.5 units lower in 150 mM Gu-HCl compared to the values in 150 mM KCl. Acid titration of wild-type bR in the presence of 150 mM Gu-HCl also shows that the pK_a of the purple-to-blue membrane conversion (or D85) is lowered from ~2.5 to ~1.9 (data not shown).

The pK_a of E204 in the M state

Fig. 8 *A* shows the flash-induced absorbance changes of the pH-sensitive dye pyranine at neutral pH in KCl and Gu-HCl (both 150 mM). As mentioned above, in 150 mM KCl at pH

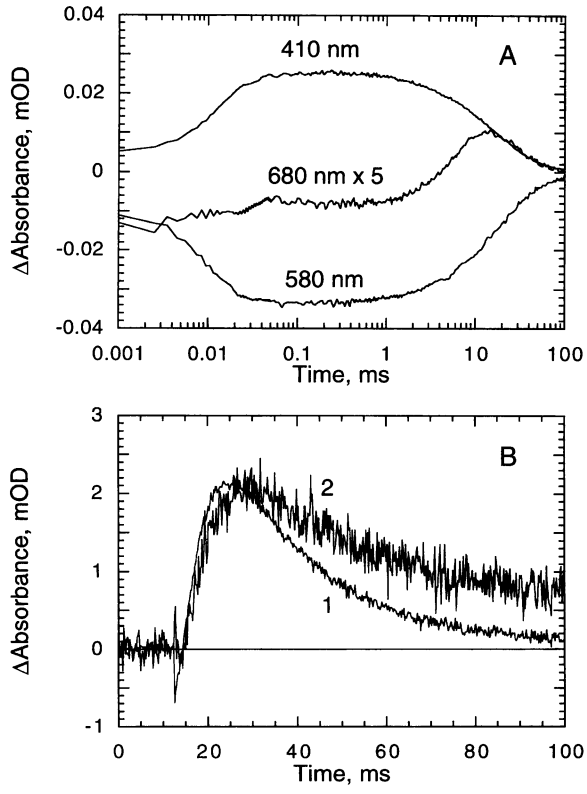

FIGURE 5 Flash-induced absorption changes in the K129H pigment. (*A*) Absorbance changes at 410 nm, 680 nm, and 580 nm. M decay (monitored at 410 nm) shows three lifetimes, $\tau_1 \approx 5$ ms (~15%), $\tau_2 \approx 17$ ms (>75%), and $\tau_3 \approx 80$ ms (<10%); the major fraction of M decay coincides with O decay (680 nm) and pigment recovery (580 nm). (*B*) Absorbance changes of the O intermediate at 680 nm (*curve 1*) and ΔA pyranine at 460 nm (*curve 2*). Both traces have been normalized at ΔA_{max} to show that proton uptake approximately coincides with O formation. $\lambda_{actinic}$ = 532 nm, 20°C, sample OD at λ_{max} = 0.25, pH = 7.0.

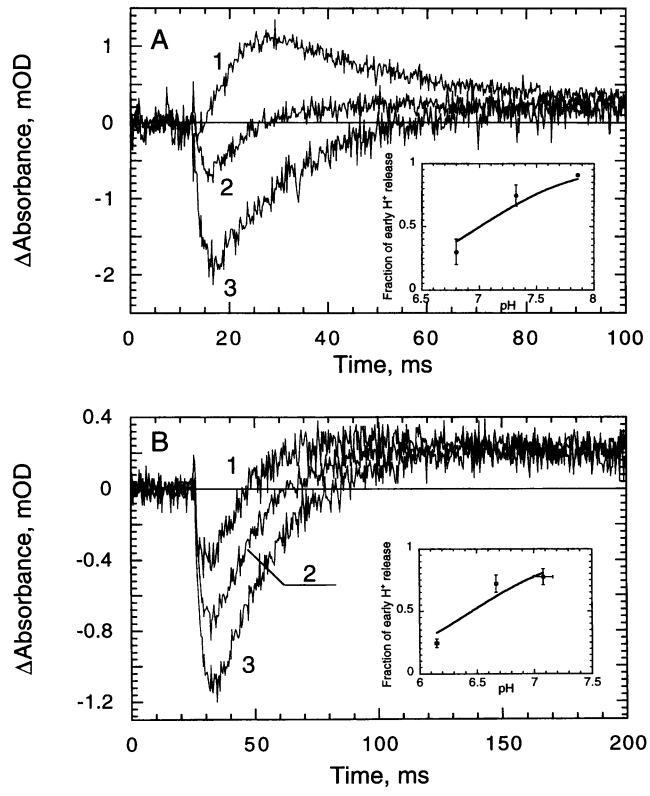

FIGURE 6 Flash-induced proton release and uptake in the K129H pigment as detected with the pH sensitive dye, pyranine. (*A*) In 150 mM KCl at pH 6.8 (*curve 1*), 7.3 (*curve 2*), and 7.9 (*curve 3*). (*B*) In 1 M NaCl/25% glycerol at pH 6.2 (*curve 1*), 6.7 (*curve 2*), and 7.0 (*curve 3*). Insets show the pH dependence of early proton release with pK_as ≈ 7.0 and 6.4 in 150 mM KCl and 1 M NaCl, respectively. $\lambda_{actinic}$ = 532 nm, 20°C, sample OD at λ_{max} = 0.25.

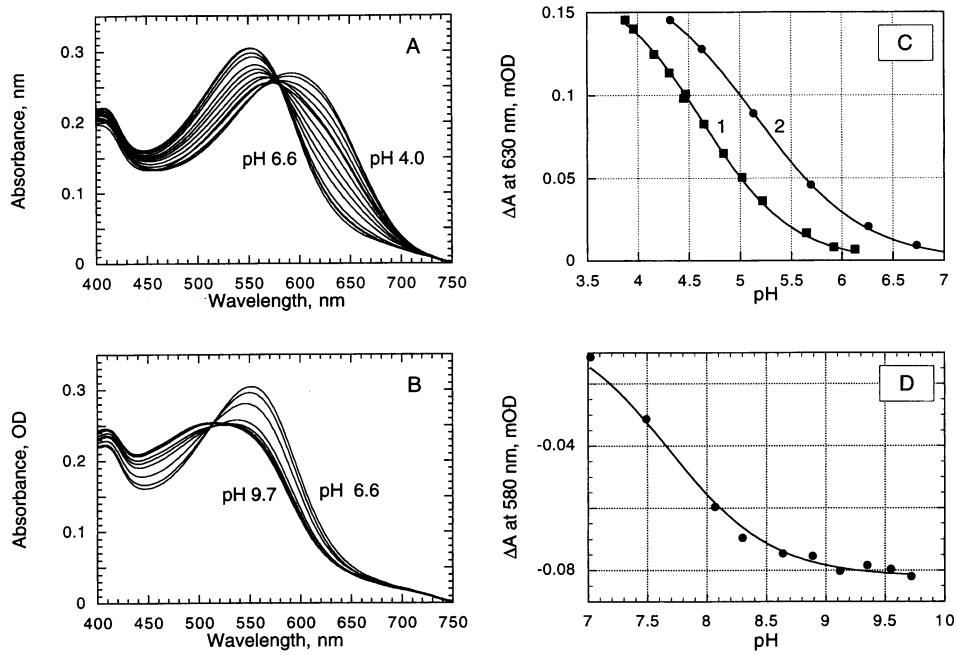

FIGURE 7 pH dependence of the absorption spectrum of the K129H pigment in 150 mM guanidine hydrochloride at 20°C. (*A*) Absorption spectra taken at pH 6.6, 6.1, 5.9, 5.2, 5.1, 5.0, 4.8, 4.7, 4.5, 4.4, 4.3, 4.2, and 4.0 showing the purple-to-blue membrane transition. (*B*) Absorption spectra taken at pH 6.6, 7.0, 7.5, 8.1, 8.3, 8.6, 8.9, 9.1, 9.4, 9.6, and 9.7 showing the alkaline transformation of the pigment into P480. (*C*) Curve 1 is ΔAbsorbance of K129H at 630 nm in 150 mM guanidine hydrochloride as a function of pH, calculated as a difference of absorbance between pH_i and pH 6.6 (absolute spectra are shown in Fig. 7 *A*). Curve 2 is ΔAbsorbance of K129H at 630 nm in 150 mM KCl as a function of pH calculated as a difference of absorbance between pH_i and pH 7.0 (absolute spectra are shown in Fig. 2 *A*). To account for the difference in absorbance of the two samples, data for curve 2 were multiplied by 0.564. The solid line represents the fit of the data with the equation $f = \text{const}/(1 + 10^{n(pK_a - pH)})$. The pK_a of the purple-to-blue membrane transition in 150 mM Gu-HCl is ~4.6 ± 0.2, $n = 0.7$ and in 150 mM KCl $pK_a = 5.1$ and $n = 0.7$ (for simplicity, the complex titration curves were fit with a single component, which led to an $n < 1$). (*D*) pH dependence of ΔAbsorbance at 580 nm (pH_i minus pH 6.6) (absolute spectra are shown in Fig. 7 *B*). pK_a of formation of P480 is ~7.7 ± 0.1, $n = 1$.

6.8, the proton release and uptake sequence is reversed compared to wild-type bR, and mostly late proton release is observed; only ~25-30% of the protons are released early. In 150 mM Gu-HCl, however, the normal order of proton release before uptake is restored and nearly 100% early proton release is observed. Fig. 8 *B* shows flash-induced proton changes (Δ*A* pyranine) at pH 6.8 in the presence of varying concentrations of guanidine hydrochloride, with the total salt concentration held constant at 150 mM. In the presence of 10 mM Gu-HCl (plus 140 mM KCl) the fraction of early proton release is ~0.7 (compared to only 0.25–0.3 early proton release seen in 150 mM KCl; see Fig. 6 *A*). In 50 mM Gu-HCl (plus 100 mM KCl) and 100 mM Gu-HCl (plus 50 mM KCl) the fractions of early proton release are ~0.9 and ~0.94, respectively. At concentrations of Gu-HCl above 100 mM, the proton signal becomes progressively smaller, until at ~0.5 M almost no proton changes are observed, possibly because Gu-HCl acts to denature the pigment at these higher concentrations. An Eadie-Hofstee plot of early proton release versus early proton release/[Gu-HCl] suggests that the dissociation constant of Gu-HCl is ~4 mM (Fig. 8 *C*); the linearity of the data suggests that approximately one guanidinium ion is bound per bR. The binding of the single guanidinium ion has the effect of lowering the apparent pK_a of E204 in M to a value of at least ~5.8 from ~7, because >90% early proton release is observable at pH 6.8 at saturating levels of Gu-HCl. The effects of guanidinium ion can be reversed by repeated washing in water.

DISCUSSION

K129 is one of the residues present in the extracellular loop connecting the transmembrane helices D and E (Grigorieff et al., 1996). It is conserved in all of the known proton pumping retinal proteins from *Halobacteria*, with the exception of archaerhodopsin-1 (aR-1), which has a histidine residue in the position analogous to K129 in bR (Mukohata et al., 1991). Although one might not expect much influence of a surface residue on the functioning of bR, we find that the substitution of K129 with histidine affects the pK_a's of two key residues in the proton transport pathway, D85 and E204, in the unphotolyzed pigment. It also increases the pK_a of E204 in the M state (relative to the WT value), causing a reversal of the order of proton release and uptake at neutral pH; the normal order of proton release followed by uptake is restored at high pH (or at high ionic strength, which has the effect of raising the pH at the surface of the membrane) or at lower pH values in the presence of moderate (millimolar) concentrations of guanidine hydrochloride.

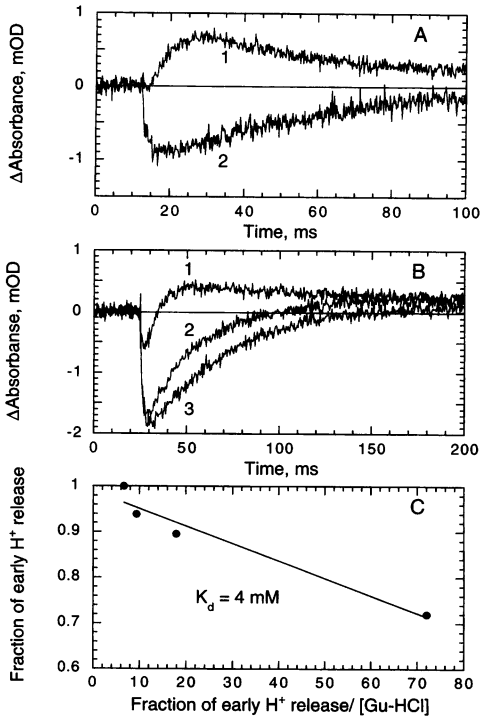

FIGURE 8 Flash-induced proton release and uptake in K129H pigment (as detected with pyranine) at pH 6.8. (*A*) In 150 mM KCl (*curve 1*) showing mostly proton uptake before release, and in 150 mM guanidine hydrochloride (*curve 2*), showing proton release before uptake. (*B*) Proton release and uptake at varying concentrations of the guanidinium ion (total salt concentration maintained at 150 mM). *Curve 1*: In 10 mM Gu-HCl (plus 140 mM KCl). *Curve 2*: In 50 mM Gu-HCl (plus 100 mM KCl). *Curve 3*: In 100 mM Gu-HCl (plus 50 mM KCl). (*C*) Eadie-Hofstee plot of fraction of early proton release as a function of fraction of early proton release/[Gu-HCl]. $K_d \approx 4$ mM, suggesting that one guanidinium ion is bound per bR. Conditions are as in Fig. 6.

pK_a of E204 in the M state (pH dependence of the fraction of early proton release)

In wild-type bR the order of proton release and uptake is reversed at low pH when the pH is below the pK_a of E204 (Dencher and Wilms, 1975; Zimányi et al., 1992). Thus a simple explanation for the reversal of the order of proton release and uptake in the K129H mutant, near neutral pH, is that the pK_a of E204 in the M state is higher than the pH of the medium (Zimányi et al., 1992; Cao et al., 1993; Govindjee et al., 1996). An alternative explanation could maintain that the pK_a of E204 is close to that in the WT (~5.8), and that the reversed order of proton release and uptake at neutral pH in K129H is instead due to differences in surface charge between K129H and the wild type. Because the intrinsic pK_a of histidine is lower than that of lysine (6.5 and 10, respectively), the overall charge on the membrane surface might be more negative in the K129H mutant than in wild-type bR, around neutral pH and up to ~pH 10. This would result in a higher concentration of protons and thus a lower pH close to the membrane surface of K129H, possibly lower than the pK_a of E204 in the M state (~5.8), making it impossible for the latter to deprotonate. One argument against this explanation is that if the surface pH is lower than 5.8, the pK_a of E204 in the M state, histidine, whose intrinsic pK_a is ~6.5, would be expected to be protonated. The surface charge would be the same as that of the wild-type pigment; hence, the argument is self-contradictory. In addition, this argument implies that if the surface charges are screened at sufficiently high ionic strength, only early proton release should be observed at neutral pH. As seen in Fig. 6 *B*, at high ionic strength (1 M salt) late proton release is not abolished; the pK_a of early proton release is only shifted to a lower value, 6.4 in 1 M salt versus ~7.0 in 150 mM salt, suggesting that the late proton release is not due to a difference in the surface charge between K129H and the WT pigment, but reflects a real difference in the pK_a's of E204 in the M states of the respective pigments. Moreover, Alexiev et al. (1994a) report that the K129C mutation does not seem to alter the measured surface charge of the purple membrane.

Interestingly, in both the archaerhodopsins, aR-1 and aR-2, there are at least four or five (if histidine is deprotonated) more negative charges on the extracellular surface than in bR (Mukohata et al., 1991); yet the aR-1 and aR-2 pigments differ in their sequence of light-induced proton release and uptake at neutral pH in 150 mM salt. aR-1, which has a histidine in the position analogous to K129 in bR, shows late proton release, which is reversed at high salt concentration, whereas in aR-2 the residue remains a lysine and the order of proton release and uptake is similar to that in bR (Lukashev et al., 1994). This again suggests that the reversal of the order of proton release and uptake in K129H at low ionic strength is not due to a change in the surface charge, but rather to specific changes in the pK_a of the proton release group, E204.

We therefore suggest that the reversal of the order of proton release and uptake is due to the effect of the substitution of K129 by histidine on the pK_a of E204 in the M intermediate, which is increased from 5.8 in the WT (Zimányi et al., 1992) to ~7.0 in K129H (at 150 mM KCl). In 1 M salt, the pK_a change upon mutation is from 4.7 in WT (Cao et al., 1993) to ~6.4.

Alteration of coupling between D85 and E204

Balashov et al. (1993, 1995, 1996) proposed a model of interacting residues whose protonation states are coupled, D85 and X′, to explain the biphasic pH dependence of the protonation of D85. Subsequent experimental evidence suggests strongly that X′ should be identified as the proton release group E204 (Brown et al., 1995; Richter et al., 1996; Govindjee et al., 1996). According to the model, the pK_a of D85 depends on the protonation state of E204 and vice versa. The change in the pK_a of E204 when D85 changes its protonation state provides a measure of the coupling between the two residues. In unphotolyzed wild-type bR, the pK_a of D85 increases considerably (by ~4.9 pH units) upon

deprotonation of E204; likewise, the pK_a of E204 decreases by ~4.9 units upon protonation of D85, suggesting a strong coupling between these two residues (Balashov et al., 1996). This coupling is substantially weaker in K129H. The fit of the dark adaptation data (Fig. 1 *A*) shows that in the unphotolyzed state, the pK_a of D85 increases by only 1.5 units from ~4.9 to 6.4 when E204 deprotonates, and the pK_a of E204 decreases by ~1.5 units from ~8.0 to ~6.5 when D85 is protonated. Similar relationships can also be deduced from the fit of D85 titration (Fig. 2 *C*). The change in the pK_a is much smaller in the K129H mutant than in the WT (1.5 versus 4.9 units), suggesting that the coupling between D85 and E204 is weaker in K129H than in wild-type bR. Interestingly, the pH dependence of dark adaptation and D85 titration is close to that in the R82K mutant (Balashov et al., 1995), in which the coupling is also reduced and pK_a's of D85 and X′ (E204) in the unphotolyzed state are closer to each other than in the WT. Thus two quite different mutations cause similar shifts in the pK_a's of D85 and E204.

It is possible that the K129H mutation affects the H-bonding network that couples E204 and D85 (Humphrey et al., 1994; Richter et al., 1996) and thus weakens the coupling between these two residues, resulting in an increase in the pK_a of D85 and a decrease in the pK_a of E204 in the unphotolyzed pigment. Alternatively, the conformation of the D-E loop in K129H pigment may be altered, resulting in a partial hydration of E204 and causing a decrease in its pK_a.

One consequence of reduced coupling between D85 and E204 is that the pK_a of E204 does not decrease to the same extent as in the WT upon protonation of D85 during the photocycle. In K129H the pK_a of E204 is ~8.1 in the unphotolyzed state and ~7.0 in the M state (in 150 mM salt). Thus the pK_a of E204 decreases by only ~1.1 pH units during the photocycle in K129H mutant versus ~3.7 pH units (from 9.5 to 5.8) in wild-type bR. This probably partially explains the small proton signals observed in K129H pigment. In K129H, the pK_a of E204 when D85 is protonated in the unphotolyzed pigment (in the blue membrane, see Fig. 2 *C*) is 0.5 units lower than when D85 is protonated in the M state (6.5 and 7.0, respectively). A difference in the pK_a of E204 in these two states was also reported for the wild-type bR, R82Q, and R82K pigments (Balashov et al., 1995, 1996; Govindjee et al., 1996) and probably reflects the influence of the protonation state of the Schiff base and isomeric state of the retinal, which differ in the two cases, on the pK_a of E204.

Correlation of M amplitude and P480 with the deprotonation of E204 in the unphotolyzed pigment

Although the nature of P480 is not certain, our initial experimental evidence suggests that, in the K129H mutant, P480 appears to be formed from the all-*trans* pigment rather than the 13-*cis* pigment. This explains the blue-shifted λ_{max} of the dark-adapted pigment at pH 8.9 compared to pH 7.0 (see Fig. 3, *A* and *B*). As shown in Fig. 3 *C* (*curve 2*), the light-adapted minus dark-adapted difference spectrum at pH 8.9 seems to be a superposition of contributions from two processes: 1) a red shift of the absorption spectrum and an increase in the extinction coefficient of the pigment due to 13-*cis* to all-*trans* isomerization, and 2) the formation of P480, because there is an increase in absorbance around 450–470 nm (Fig. 3 *C*, *curve 2*).

There is a correlation between the decrease in the amplitude of the M intermediate and a decrease in the absorbance of the purple pigment (and the increase in P480). The fractional decreases in the M amplitude (Fig. 4 *A*) and in the amount of purple pigment (Fig. 2 *D*) are similar, and the two processes have similar pK_as, suggesting a common origin. Thus a simple explanation for the decrease in the M amplitude at high pH, in the K129H mutant, is that the pigment is transformed into P480, which does not yield a 410-nm photoproduct.

We propose that the alkaline transformation of the pigment into P480 reflects the deprotonation of some group at high pH. Because the pK_a of P480 formation (Fig. 2 *D*) matches the high pK_a transition in the titration of the dark-adaptation rate constant (Fig. 1 *A*), it is likely that deprotonation of E204 is responsible for both events. As noted above, the decrease in the M amplitude (Fig. 4 *A*) has a similar pK_a, suggesting that the deprotonation of E204, which leads to the partial transformation of the purple pigment into P480, is also the source of the decrease in the M amplitude. In addition, in E204Q the relatively constant amplitude of the M intermediate over a wide pH range, from pH 5.6 to 10.5 (Fig. 4 *A*), and the absence of an alkaline transition of the pigment into P480 (Fig. 4 *B*) also suggest that the deprotonation of E204 is responsible for the decline in the M amplitude due to the transformation of the pigment into the P480 species in K129H mutant. The fact that the transition between the purple pigment and P480 does not go to completion (Fig. 2 *A*), and that the M amplitude does not go to zero but instead levels off at high pH, suggests that the deprotonation of E204 is not itself directly responsible for P480 formation. That is, the deprotonation of E204 is not sufficient for transformation to P480. If E204 were the sole determinant of P480 formation, 100% of the pigment would be expected to transform into P480 with increasing pH, in a manner consistent with a traditional titration curve. Whether the correspondence between the formation of P480, the deprotonation of E204, and the decrease in M amplitude will hold for other pigments remains to be investigated.

pK_a of D85

Substitution of K129 with histidine has a dramatic influence on the pK_a of D85, which increases from ~2.6 in the WT bR to ~5.1 in the K129H pigment (Table 1). An effect of an alteration in the surface charge on the pK_a of D85 can be ruled out by the arguments made above. Thus the increase

in the pK_a of D85 in K129H is most probably due to the specific substitution of K129 with histidine, suggesting that this surface residue can influence the pK_as of residues in the retinal binding pocket. It is noteworthy that the aR-1 and aR-2 pigments also show slightly elevated pK_as for the blue-to-purple membrane transition, but the different surface charge in those pigments may contribute to this (Mukohata et al., 1991). As mentioned above, it is possible that the effect of the K129H substitution on the pK_a of D85 may be indirectly mediated via E204. That is, the decrease in the pK_a of E204, because of altered protein conformation or altered hydrogen bonding in the K129H pigment, may increase the pK_a of D85, consistent with the coupling between these two residues.

Effects of guanidinium ions

Guanidine hydrochloride is commonly used as an agent for denaturing proteins. The concentrations used for such purposes, however, are typically in the molar range. We show that Gu-HCl, when used at moderate concentrations (millimolar range), can counteract the alteration of the proton release process caused by the K129H mutation and restore the normal order of proton release before uptake at neutral pH. A much smaller fraction of early proton release is observed at an equal concentration of KCl. When the total salt concentration is kept constant, the fraction of early proton release increases with increasing concentration of Gu-HCl (see Fig. 8), in a manner suggesting binding of Gu-HCl to bR in a 1:1 ratio.

Restoration by guanidinium of the normal order of proton release before uptake was shown in the R82A mutant (Alexiev et al., 1996), which otherwise shows a reversed order of proton release and uptake at neutral pH. Gu-HCl also lowers the pK_a of D85 in the R82 mutants (Alexiev et al., 1996; Renthal and Chung, 1996). It has been shown to restore the chloride pump inactivated by the mutation of R108 in halorhodopsin (Rüdiger et al., 1996), where it was suggested that it substitutes for the guanidinium group of the mutated arginine. In all of these cases, however, the mutated residue is an arginine; in these cases, one might suggest that Gu-HCl functions simply by replacing the missing guanido group. In K129H we show that Gu-HCl has an effect even though the mutation does not involve replacement of an arginine. Additionally, in wild-type bR, Gu-HCl also lowers the pK_a of D85 from 2.5 to $\sim$1.9 $\pm$ 0.1 (in 150 mM KCl/25% glycerol; data not shown).

The effect of Gu-HCl could be mediated in two different ways. The guanidinium ion may act as a more efficient screener of the membrane surface charge than small monatomic cations such as K^+. It is possible that the guanidinium ion is able to partition near or into the membrane surface more easily, and thus efficiently screen the negative charges on the membrane surface, raising the surface pH more than an equivalent concentration of monatomic cations and causing an apparent decrease in the measured pK_a's of intramembrane protonable groups. Alternatively, guanidinium may bind to a specific site on bR, probably in the extracellular half of the protein, and directly or indirectly perturb the pK_a's of D85 and E204 (in the unphotolyzed pigment and in the M state).

Are the effects of K129 modification substituent-specific?

Three lines of evidence, namely the present experiments on K129H, the studies of aR-1 in which there is an histidine at the 129 position, and the effects of chemical modifications of lysines in wild-type bR (Takeuchi et al., 1981; Singh and Sonar, 1988; Li, Govindjee and Ebrey, unpublished observations), show that alteration of K129 affects the pK_a's of D85 and E204. It is puzzling, however, that neither the covalent attachment of fluorescein to K129 (Heberle and Dencher, 1990, 1992; Scherrer et al., 1992) nor the mutation of K129 to a cysteine, and the subsequent modification of that cysteine by the attachment of either iodoacetamidofluorescein (Scherrer et al., 1992; Alexiev et al., 1994b) or a spin label (Altenbach et al., 1990), appears to alter the properties of the resulting pigment from those of WT bR. In addition, the current high-resolution structure of bR (Grigorieff et al., 1996) places K129 at a position relatively far (>12 [angts]A) away from E204, reducing the possibility of a direct electrostatic interaction between the two residues. It thus appears that the effects of K129 modification are not merely due to the elimination of the putative positive charge of the lysine, but may rather depend in a complex manner on the identity of the substituent. The perturbation caused by a particular substituent must be mediated through the relatively large distance between K129 and E204 through a suitable mechanism. Three such mechanisms, varying in scale, can be suggested.

One possibility is that the determining factor is the hydrophobicity of the substituent. Fluorescein has several negative charges, suggesting that it would protrude into the solvent rather than partition into the membrane when covalently bound to K129 (or C129). Acetylation or modification by fluorescamine, however, removes the charge of the lysine without contributing any additional charge. Histidine in its uncharged form is similarly more hydrophobic than lysine, although it is not clear whether the same relationship holds with cysteine. A hydrophobic group at the 129 position might associate more with the protein interior, possibly disrupting the hydrogen bonding and water structure in the extracellular half of the protein channel and thus perturbing E204. In contrast, a hydrophilic group at the same position might be oriented into the solution, away from the membrane and the protein.

A second possibility, not wholly distinct from the first, is that a substituent may cause a perturbation in the structure of the entire D-E loop, leading to rearrangements of bound waters throughout the extracellular half of the protein and affecting the pK_a's of E204 and other charged groups. The

rearrangement of the loop could be caused by the different partitioning of the substituent mentioned in the previous paragraph, which would have the effect of "tugging" the loop toward one conformation or another. Alternatively, a loop rearrangement could be caused by different steric requirements of particular substituents.

Finally, the perturbation caused by a particular substituent could lead to a macroscopic change in the protein conformation not limited to the D-E loop. In this case, it is likely that both D85 and E204 would be affected because of the global nature of such a change, not merely because of their interaction with each other. Such a perturbation could involve a change in helix tilts, or some "melting" or disruption of the D and E helices. Considering the ability of the K129H protein to pump protons, and the not unusual absorbance and photocycling properties of the pigment, a large-scale disruption of the structure appears unlikely, and a local perturbation at position 129 or at the D-E loop as a whole provides a more plausible explanation for the effects we have observed. In both of these cases, the effects are ostensibly transduced from the 129 position to E204 by disruption of a proposed bound water network in the extracellular half of the protein (Humphrey et al., 1994) and/or steric or conformational changes that disrupt the charge environment around E204.

We wish to thank Burr Nelson for providing the various computer programs used in the data analysis, and Patrice Goletz for the mutant membranes.

This work was supported by National Institutes of Health grant GM52023 (to TGE) and by National Institutes of Health grant EY04939 and DOE grant 95ER20171 (to RKC).

REFERENCES

Alexiev, U., M. P. Krebs, R. Mollaaghababa, H. G. Khorana, and M. P. Heyn. 1996. Restoring the photocycle and proton pumping in the mutant R82A of bacteriorhodopsin by guanidinium hydrochloride or a second mutation on the cytoplasmic surface. *Biophys. J.* 70:A107.

Alexiev, U., T. Marti, M. P. Heyn, H. G. Khorana, and, P. Scherrer. 1994a. Surface charge of bacteriorhodopsin detected with covalently bound pH indicators at selected extracellular and cytoplasmic sites. *Biochemistry.* 33:298–306.

Alexiev, U., T. Marti, M. P. Heyn, H. G. Khorana, and P. Scherrer. 1994b. Covalently bound pH-indicator dyes at selected extracellular or cytoplasmic sites in bacteriorhodopsin. 2. Rotational orientation of helices D and E and kinetic correlation between M formation and proton release in bacteriorhodopsin micelles. *Biochemistry.* 33:13693–13699.

Altenbach, C., T. Marti, H. G. Khorana, and W. L. Hubbell. 1990. Transmembrane protein structure: spin labelling of bacteriorhodopsin mutants. *Science.* 248:1088–1092.

Balashov, S. P., R. Govindjee, and T. G. Ebrey. 1991. Red shift of the purple membrane absorption band and the deprotonation of tyrosine residues at high pH. *Biophys. J.* 60:475–490.

Balashov, S. P., R. Govindjee, E. S. Imasheva, S. Misra, T. G. Ebrey, Y. Feng, R. K. Crouch, and D. R. Menick. 1995. The two pK_a's of aspartate-85 and control of thermal isomerization and proton release in the arginine-82 to lysine mutant of bacteriorhodopsin. *Biochemistry.* 34:8820–8834.

Balashov, S. P., R. Govindjee, M. Kono, E. Imasheva, E. Lukashev, T. G. Ebrey, R. K. Crouch, D. R. Menick, and Y. Feng. 1993. Effect of the arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle. *Biochemistry.* 32: 10331–10343.

Balashov, S. P., E. S. Imasheva, R. Govindjee, and T. G. Ebrey. 1996. Titration of aspartate-85 in bacteriorhodopsin: what it says about chromophore isomerization and proton release. *Biophys. J.* 70:473–481.

Brown, L. S., J. Sasaki, H. Kandori, A. Maeda, R. Needleman, and J. K. Lanyi. 1995. Glutamic acid 204 is the terminal proton release group at the extracellular surface of bacteriorhodopsin. *J. Biol. Chem.* 270: 27122–27126.

Cao, Y., L. S. Brown, R. Needleman, and J. K. Lanyi. 1993. Relationship of proton uptake on the cytoplasmic surface and the reisomerization of the retinal in the bacteriorhodopsin photocycle: an attempt to study the complex kinetics of the protons and the N and O intermediates. *Biochemistry.* 32:10239–10248.

Dencher, N., and M. Wilms. 1975. Flash photometric experiments on the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mech.* 1:259–271.

Druckmann, S., M. Ottolenghi, A. Pande, J. Pande, and R. H. Callender. 1982. Acid-base equilibrium of the Schiff base in bacteriorhodopsin. *Biochemistry.* 21:4953–4959.

Ebrey, T. G. 1993. Light energy transduction in bacteriorhodopsin. *In* Thermodynamics of Membranes, Receptors and Channels. M. Jackson, editor. CRC Press, Boca Raton, FL. 353–387.

Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211–230.

Govindjee, R., S. P. Balashov, and T. G. Ebrey. 1990. Quantum efficiency of the photochemical cycle of bacteriorhodopsin. *Biophys. J.* 58: 597–608.

Govindjee, R., M. Kono, S. P. Balashov, E. Imasheva, M. Sheves, and T. G. Ebrey. 1995. Effects of substitution of tyrosine 57 with asparagine and phenylalanine on the properties of bacteriorhodopsin. *Biochemistry.* 34:4828–4838.

Govindjee, R., E. Lukashev, M. Kono, S. P. Balashov, T. G. Ebrey, J. Soppa, J. Tittor, and D. Oesterhelt. 1992. Tyr57Asn mutant of bacteriorhodopsin: M formation and spectral transformations at high pH. *In* Structures and Functions of Retinal Proteins. J. L. Rigaud, editor. John Libbey Eurotext, Montrouge, France. 115–118.

Govindjee, R., S. Misra, S. P. Balashov, T. G. Ebrey, R. Crouch, and D. R. Menick. 1996. Arginine-82 regulates the pK_a of the group responsible for the light-driven proton release in bacteriorhodopsin. *Biophys. J.* 71:1011–1023.

Grigorieff, N., T. A. Ceska, K. H. Downing, J. M. Baldwin, and R. Henderson. 1996. Electron-crystallographic refinement of the structure of bacteriorhodopsin. *J. Mol. Biol.* 259:393–421.

Heberle, J., and N. A. Dencher. 1990. Bacteriorhodopsin in ice: accelerated proton transfer from the purple membrane surface. *FEBS Lett.* 277: 277–280.

Heberle, J., and N. A. Dencher. 1992. Surface-bound probes monitor proton translocation and surface potential changes during the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 89:5996–6000.

Humphrey, W., I. Logunov, K. Schulten, and M. Sheves. 1994. Molecular dynamics study of bacteriorhodopsin and artificial pigments. *Biochemistry.* 33:3668–3678.

Khorana, H. G. 1993. Two light-transducing membrane proteins: bacteriorhodopsin and the mammalian rhodopsin. *Proc. Natl. Acad. Sci. USA.* 90:1166–1171.

Kono, M., S. Misra, and T. G. Ebrey. 1993. pH dependence of light-induced proton release by bacteriorhodopsin. *FEBS Lett.* 331:31–34.

Lanyi, J. K. 1993. Proton translocation mechanism and energetics in the light-driven pump bacteriorhodopsin. *Biochim. Biophys. Acta Bioenerg.* 1183:241–261.

Lukashev, E. P., R. Govindjee, M. Kono, T. G. Ebrey, Y. Sugiyama, and Y. Mukohata. 1994. pH dependence of the absorption spectra and photochemical transformations of the archaerhodopsins. *Photochem. Photobiol.* 60:69–75.

Metz, G., F. Siebert, and M. Engelhard. 1992. Asp85 is the only residue that gets protonated in the M intermediate and the purple to blue transition of bacteriorhodopsin. A solid state ^{13}C CP-MAS NMR investigation. *FEBS Lett.* 303:237–242.

Mowery, P. C., R. H. Lozier, Q. Chae, Y.-W. Tseng, M. Taylor, and W. Stoeckenius. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry.* 18:4100–4107.

Mukohata, Y., K. Ihara, K. Uegaki, Y. Miyashita, and Y. Sugiyama. 1991. Australian *Halobacteria* and their retinal-protein ion pumps. *Photochem. Photobiol.* 54:1039–1045.

Oesterhelt, D., J. Tittor, and E. Bamberg. 1992. A unifying concept for ion translocation by retinal proteins. *J. Bioenerg. Biomembr.* 24:181–191.

Otto, H., T. Marti, M. Holz, T. Mogi, L. J. Stern, F. Engle, H. G. Khorana, and M. P. Heyn. 1990. Substitution of amino acids Asp-85, Asp-212, and Arg-82 in bacteriorhodopsin affects the proton release phase of the pump and the pK of the Schiff base. *Proc. Natl. Acad. Sci. USA.* 87:1018–1022.

Renthal, R., and Y. J. Chung. 1996. Guanidinium restores the purple chromophore of bacteriorhodopsin in the R82Q mutant. *Biophys. J.* 70:A109.

Richter, H.-T., L. S. Brown, R. Needleman, and J. K. Lanyi. 1996. A linkage of the pK_a's of Asp-85 and Glu-204 forms part of the reprotonation switch of bacteriorhodopsin. *Biochemistry.* 35:4054–4062.

Rothschild, K. J. 1992. FTIR difference spectroscopy of bacteriorhodopsin: toward a molecular model. *J. Bioenerg. Biomembr.* 24:147–167.

Rüdiger, M., U. Haupts, K. Gerwert, and D. Oesterhelt. 1996. Chemical reconstitution of a chloride pump inactivated by a single point mutation. *EMBO J.* 14:1599–1606.

Sampogna, R. V., and B. Honig. 1994. Environmental effects on the protonation states of active site residues in bacteriorhodopsin. *Biophys. J.* 66:1341–1352.

Sampogna, R. V., and B. Honig. 1996. Electrostatic coupling between retinal isomerization and the ionization state of Glu-204: a general mechanism for proton release in bacteriorhodopsin. *Biophys. J.* 71: 1165–1171.

Scharnagl, C., J. Hettenkofer, and S. F. Fischer. 1994. Proton release pathway in bacteriorhodopsin: molecular dynamics and electrostatic calculations. *Int. J. Quantum Chem.* S21:33–56.

Scharnagl, C., J. Hettenkofer, and S. F. Fischer. 1995. Electrostatic and conformational effects on the proton translocation steps in bacteriorhodopsin: analysis of multiple M structures. *J. Phys. Chem.* 99:7787–7800.

Scherrer, P., U. Alexiev, H. Otto, M. P. Heyn, T. Marti, and H. G. Khorana. 1992. Proton movement and surface charge in bacteriorhodopsin detected by selectively attached pH indicators. *In* Structures and Functions of Retinal Proteins. J. L. Rigaud, editor. John Libbey Eurotext, Montrouge, France. 205–211.

Singh, A. K., and S. M. Sonar. 1988. Chemical modification of bacteriorhodopsin by fluorescamine. *Biochim. Biophys. Acta.* 955:261–268.

Subramaniam, S., T. Marti, and H. G. Khorana. 1990. Protonation state of Asp (Glu)-85 regulates the purple-to-blue transition in bacteriorhodopsin mutants Arg-82→Ala and Asp-85→Glu: the blue form is inactive in proton translocation. *Proc. Natl. Acad. Sci. USA.* 87:1013–1017.

Sugiyama, Y., M. Maeda, M. Futai, and Y. Mukohata. 1989. Isolation of a gene that encodes a new retinal protein, archaerhodopsin, from *Halobacterium* sp. aus-1. *J. Biol. Chem.* 264:20859–20862.

Takeuchi, Y., K. Ohno, M. Yoshida, and K. Nagano. 1981. Light-induced proton dissociation and association in bacteriorhodopsin. *Photochem. Photobiol.* 37:587–592.

Uegaki, K., Y. Sugiyama, and Mukohata. 1991. Archaerhodopsin-2, from *Halobacterium* sp. aus-2 further reveals essential amino acid residues for light-driven proton pumps. *Arch. Biochem. Biophys.* 286:107–110.

Zimányi, L., G. Váró, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi. 1992. Pathways of proton release in the bacteriorhodopsin photocycle. *Biochemistry.* 31:8535–8543.