Reorientations in the Bacteriorhodopsin Photocycle Are pH Dependent

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ABSTRACT Chromophore reorientations during the bacteriorhodopsin photocycle in the purple membrane of *Halobacterium salinarium* have been detected by time-resolved linear dichroism measurements of the optical anisotropy over the pH range from 4 to 10 and at ionic strengths from 10 mM to 1 M. The results show that reorientations in the L and M states of bacteriorhodopsin are pH dependent, reaching their largest amplitude when the membrane is at pH 6–8. Reorientations on the millisecond time scale of unexcited spectator proteins in the native purple membrane also depend on pH, consistent with the suggestion that spectator reorientations are triggered by reorientation of the photoexcited protein. The results imply that a group with a pK_a of 5 to 6 enables reorientations, and that the deprotonation of a site at pH values above 9 restricts reorientational motion. This suggests that reorientations in M may be correlated with proton release.

INTRODUCTION

The structure of the membrane protein bacteriorhodopsin (BR) has been characterized by electron diffraction (Henderson et al., 1990), stimulating interest in structure-function correlations as a model for proton pumping, as well as for the function of visual proteins and other rhodopsin-like proteins. Although several features of bacteriorhodopsin's proton pumping mechanism are known (Lanyi, 1993; Ebrey, 1993), essential aspects of the structure-function relationship in bacteriorhodopsin are still in question. Although it is clear that certain residues play a key role in the proton pump, the nature of conformational changes in the protein and their role in the proton pump are not yet well understood.

A model has emerged for proton pumping in BR (see Lanyi, 1993, and Ebrey, 1993, for reviews) in which pK_a changes in the Schiff base and residues D85 and D96 comprise essential features in the proton pumping mechanism. At physiological pH, proton release from the extracellular side is followed by proton uptake on the cytoplasmic side. The pK_as of the important functional groups are initially 9, 13, and 3 for D96, the Schiff base, and D85, respectively, in ground-state, light-adapted BR (Lanyi, 1993; Ebrey, 1993; Bashford and Gerwert, 1992; Sampogna and Honig, 1994). After the inception of the photocycle, the pK_a of the Schiff base drops while the pK_a of D85 increases (Lanyi, 1993; Braiman et al., 1995), allowing proton transfer from the Schiff base to D85 in the L $\rightarrow$ M step. During or after the L $\rightarrow$ M transition, a proton is released to the extracellular medium by a group with a pK_a of 5.8 (Zimányi et al., 1992). In the M $\rightarrow$ N transition, the pK_a of D96 decreases while the pK_a of the Schiff base increases, resulting in proton displacement from D96 to the Schiff base via a water channel (Cao et al., 1993). Finally, the pK_as return to their original levels and a proton is accepted from the cytoplasmic side by a site with a pK_a of approximately 11 (Zimányi et al., 1993).

This sequence of events raises the important question of how these events are associated with other changes in the protein or the purple membrane on a broader scale. Evidence from a variety of sources now emphatically supports suggestions that the protein changes conformation during the BR photocycle. A perturbation of Asp-96 bands appears in the L state difference Fourier transform infrared (FTIR) spectrum (Braiman et al., 1991; Maeda et al., 1992). Further changes appear in M (Sasaki et al., 1994; Takei et al., 1994) associated with Asp-96, Asp-115, and a lysine residue assigned to Lys-216 at the active site. Evidence of changes in protein structure between the BR and M states was also detected by electron (Subramaniam et al., 1993; Han et al., 1994), neutron (Dencher et al., 1989; Hauss et al., 1994), and x-ray diffraction (Koch et al., 1991; Dencher et al., 1991). Whereas FTIR difference spectra are sensitive to changes localized at specific sites, changes in electron density in the cytoplasmic side of the F and G helices (Subramaniam et al., 1993) suggest a more global conformational change.

The approach taken in our laboratory has been to measure chromophore reorientations at various stages of the photocycle (Song et al., 1994; Wan et al., 1993). Anisotropy changes corresponding to reorientational motions were observed in the L and M states, followed by a return to nearly the initial anisotropy in the O State. Recovery of the initial orientation demonstrates that the observed motions are photoinduced and reversible rather than diffusive. Although optical anisotropy measurements cannot distinguish among chromophore reorientation due to protein conformational changes, protein reorientation within the membrane, or larger scale changes in the membrane, reversible photoinduced reorientations must in any case be driven by internal changes in the protein. These observations raise the question of the role of reorientational motions in BR and their possible relationship to protein function. The intent of this paper is to explore such a relationship by determining how reorientational motions are correlated with pH. The pH

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dependence suggests that these motions may be associated in some manner with proton release to the extracellular medium.

EXPERIMENTAL PROCEDURES

Time-resolved transient absorption (TA) and time-resolved linear dichroism (TRLD) signals were detected as described previously (Song et al., 1994; Wan et al., 1993). TRLD and TA signals were measured at three probe wavelengths: 410 nm for M-state measurements, 550 nm for L-state measurements, and 550–570 nm for ground-state measurements. The TRLD, $D(t) = \Delta A_{\parallel} - \Delta A_{\perp}$; the isotropic transient absorption, $\Delta A = \Delta A_{\parallel} + 2\Delta A_{\perp}$; and the anisotropy, $r = (\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + 2\Delta A_{\perp})$, were calculated from the digitized signals.

The purple membrane was extracted from *Halobacterium salinarium* strain ET1-001 by standard methods (Oesterhelt and Stoeckenius, 1974) and suspended in a buffer solution consisting of 8.5 mM acetic acid and 1.5 mM sodium acetate at pH 4.0; 3.5 mM acetic acid and 6.5 mM sodium acetate at pH 5.0; 10 mM 2-(*N*-morpholino)ethanesulfonic acid buffer at pH 6.0; 10 mM phosphate buffer at pH 7.0; and 10 mM tris(hydroxymethyl) amino ethane buffer at pH 8.0; 10 mM boric acid at pH 9.0; 10 mM boric acid at pH 10.0; with 5 mM sodium azide. For ionic-strength studies, KCl was added to achieve the desired salt concentration. Solutions of 1 M HCl or 1 M NaOH were added dropwise until the correct pH was obtained. Low-ionic-strength samples were prepared by suspending the purple membrane in doubly distilled, deionized water at pH 7. The gel samples were prepared as described previously (Song et al., 1994).

Samples were light adapted by exposure to a 100-W lamp. Liquid samples were flowed at 25°C through a 2-mm-thick sample cell at a rate of 1 to 4 mm/s to prevent repetitive excitation and sample heating. There is no detectable flow orientation at this flow rate. The gel sample was rotated during data collection to prevent repetitive excitation and sample heating. The sample was optically clear, with an optical density of about 1 to 1.6 at 568 nm. The sample temperature for all measurements was 25°C.

RESULTS

Anisotropies were measured as the ratio $D(t)/\Delta A(t)$ over the pH range from 4 to 10. From these measurements, the anisotropies r_L, r_M, and r_{BR} of the L and M intermediates and the light-adapted ground state (BR) were determined by methods described by Song et al. (1994). Anisotropies were measured either at a time delay corresponding to the maximum absorbance of an intermediate or in scans of the time delay spanning the decay of a given intermediate. The results are collected in Fig. 1 and Table 1. For each measurement the pump power was limited, so that the absorption was not saturated.

L-state and BR ground-state anisotropies (Fig. 1 *A*) were calculated from the anisotropies measured at 550 nm from 0 to 390 μs by fitting the anisotropy decay to the equation (Song et al., 1994)

$$r_\lambda(t) = r_{BR} + \frac{\Delta A_L(t)}{\Delta A(t)}(r_L - r_{BR}). \quad (1)$$

The L-state anisotropy reaches a value of 0.38 at pH 4, 5, and 9 and drops to 0.36–0.37 at pH 7–8. These changes are small (0.02 between pH 5 and pH 7) but follow the same trend observed in the M state (Fig. 1 *B*). The value at pH 7 agrees (within experimental error) with the value reported previously (Song et al., 1994).

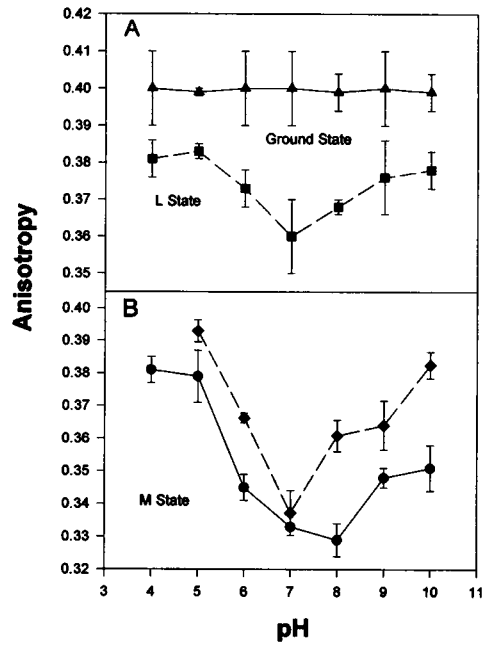

FIGURE 1 Anisotropy dependence on pH for (*A*) ground state BR (▲), the L state (■), and (*B*) the M state in 15-mM buffer (●) and in 100 mM KCl plus buffer (◆). The anisotropy of the L state was measured over time delays from of 10 to 390 μs, the anisotropy of the M state at a time delay of 390 μs, and the anisotropy of BR at 390 μs.

Fixed-time scans at 410 nm were used to collect the M-state data over the range pH 4–10 at a time delay of 390 μs. Fig. 1 *B* shows the M-state anisotropy as a function of pH in 15 mM buffer (*circles*) and in buffer with 100 mM KCl (*diamonds*). The dependence of the M-state anisotropy on pH is more pronounced than for the L state. Corrections for ground-state contributions at 410 nm are small (Song et al., 1994) and have not been incorporated. We were able to fit the pH dependence of the M-state anisotropies with pK_as of 6 and 8–9. On the high pH side, the apparent pK_a seems to shift down by up to 1

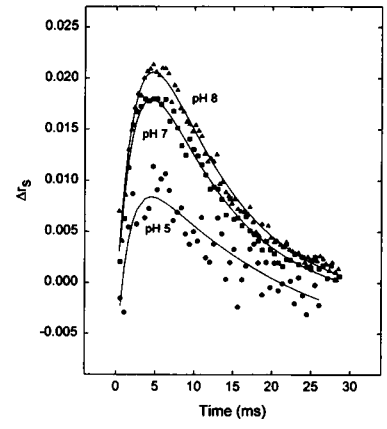

FIGURE 2 Spectator anisotropy change at pH 5.0 (●), pH 7.0 (■), and pH 8.0 (▲), calculated from TRLD data by Eq. 5 in the text. The solid lines show a least-squares fit to Eq. 6 with the fitting parameters tabulated in Table 2.

TABLE 1 pH dependence of anisotropies in the L and M intermediates

	L state (in 15 mM buffer)		M state (in 15 mM buffer)		M state (in 100 mM KCl and 15 mM buffer)	
pH	r_L	θ_L*	r_M	θ_M*	r_M	θ_M*
4.0			0.381 ± 0.004	10.1°		
5.0	0.383 ± 0.005	9.7°	0.379 ± 0.008	10.6°	0.393 ± 0.003	6.2°
6.0	0.375 ± 0.01	11.8°	0.345 ± 0.001	17.5°	0.366 ± 0.002	13.8°
7.0	0.36 ± 0.01	14.9°	0.333 ± 0.001	19.5°	0.337 ± 0.007	18.9°
8.0	0.368 ± 0.001	13.2°	0.329 ± 0.005	20.1°	0.361 ± 0.005	14.8°
9.0	0.373 ± 0.009	12.1°	0.348 ± 0.003	17.0°	0.364 ± 0.008	14.2°
10.0	0.378 ± 0.005	10.9°	0.351 ± 0.007	16.5°	0.383 ± 0.006	9.7°

Reported uncertainties are standard errors from least-squares fits.
*Reorientation angle calculated from $r_i = (3\cos^2 \theta_i - 1)/5$, with the assumption that each photocycling BR reorients identically.

unit with an increase in ionic strength from 15 mM to 115 mM. A shift of this magnitude is consistent with previous studies of the pH dependence of proton release (Kono et al., 1993).

Anisotropies were also measured for purple membrane immobilized in a polyacrylamide gel at pH 5.0 and at pH 7.0. The M-state anisotropy in the gel was 0.38 ± 0.01 at pH 5.0 and 0.35 ± 0.01 at pH 7.0. The value at pH 5.0 is consistent with the measurement of Der et al. (1988), who reported at an M-state anisotropy of 0.38 ± 0.01 at pH 5.5.

On a time scale of milliseconds, anisotropy changes have been detected and attributed to reorientational motions of spectator BR, i.e., members of the trimer adjacent to a photoexcited BR (Wan et al., 1993). The results in Fig. 2 and Table 2 show that these reorientations are also sensitive to pH. The spectator anisotropy changes were analyzed in terms of the reversible reorientation model (Wan et al., 1993), which was proposed to account for anisotropy decays at 570 nm on the millisecond time scale. These anisotropy decays cannot be explained by reorientation in photoexcited BR or by changes in overall membrane curvature, because a constant anisotropy has been observed in the M and O states on the same time scale (Song et al., 1994; Wan et al., 1993). Rather, the millisecond time-scale anisotropy decays at 570 nm can be explained by reorientations of spectator BR. In this model, the measured anisotropy is regarded as consisting of two contributions: one (r_h) from the hole created in the BR ground orientational distribution by photoexcitation, and the other (r_e) from the recovering BR population that has completed the photocycle. The key point is that at long times, any difference between the anisotropy of the hole and recovering populations is magnified by the diminishing TA signal. The TRLD contribution from ground-state BR is (Wan et al., 1993)

$$D_{BR}(t) = \Delta A_{BR}(0)[r_h(t) - r_e(t)] + r_e(t)\Delta A_{BR}(t), \quad (2)$$

where $\Delta A_{BR}(0)$ is the bleach in the ground-state BR absorption resulting from photoexcitation. The anisotropy of the hole can be written

$$r_h = r_e - \Delta r_s(t), \quad (3)$$

where $\Delta r_s(t)$ is the anisotropy change of the non-photoexcited (spectator) BR due to reorientation of spectator BR. The spectator anisotropy change can then be found from

$$\Delta r_s(t) = \left(\frac{r_e \Delta A_{BR}(t) - D_{BR}(t)}{\Delta A_{BR}(0)}\right) \quad (4)$$

We suppose in this model that the photocycling proteins return to their original orientation, so that $r_e = 0.4$, a value that is consistent with the $t \rightarrow 0$ limit of the anisotropy measured at 570 nm on the millisecond time scale. It is then possible to determine $\Delta r_s(t)$ from the experimentally determined value of $\Delta A_{BR}(0)$ and from the TA and TRLD data. Equation 4 can be used to calculate the spectator anisotropy changes $\Delta r_s(t)$ directly from the data. We have found it convenient, to reduce the uncertainty in the calculated $\Delta r_s(t)$, to fit the ground-state recovery at 570 nm to an exponential function with time constant τ_{BR}. The spectator anisotropy change is then given by

$$\Delta r_s(t) = r_e e^{-t/\tau_{BR}} - D_{BR}(t)/\Delta A_{BR}(0). \quad (5)$$

The spectator anisotropy changes calculated in this way at pH 5.0, 7.0, and 8.0 are shown in Fig. 2. The pH 4.0 data were noisy because of turbidity in the sample. The pH 9 and 10 data, which are not shown, are expected to contain significant contributions from the N state (Váró and Lanyi, 1990; Ames and Mathies, 1990).

The spectator anisotropy changes $\Delta r_s(t)$ can be fit to the reversible reorientation model (Wan et al., 1993), in which the spectator proteins rotate with a time constant τ_a and then return to their original orientation with a time constant τ_b:

$$\Delta r_s(t) = 2\Delta r_s[1 - \exp(-t/\tau_a)]\exp(-t/\tau_b). \quad (6)$$

TABLE 2 Spectator anisotropy changes and time constants

pH	τ_a* (ms)	τ_b* (ms)	Δr_s*	τ_{BR}‡ (ms)
5.0	1.7 ± 0.8	20.7 ± 9.3	0.022 ± 0.002	8.1 ± 0.3
6.0	3.2 ± 0.5	10.9 ± 0.4	0.039 ± 0.002	7.3 ± 0.4
7.0	3.0 ± 0.6	20.5 ± 1.5	0.040 ± 0.005	5.9 ± 0.3
8.0	3.43 ± 0.14	10 ± 3	0.048 ± 0.001	6.1 ± 0.1

*Obtained from fits of Eq. 6 to spectator anisotropy changes. Uncertainties are the standard errors in least-squares fits.
‡Obtained from a single-exponential fit of the TA data for recovery of BR.

TABLE 3 Dependence of anisotropies on ionic strength

Ionic strength	r_{BR}	Δr_s*	r_K	r_L	r_M
Deionized water	0.408 ± 0.002	0.013 ± 0.01	0.37 ± 0.01	0.385 ± 0.008	‡
Buffer (15 mM)	0.40 ± 0.01	0.05 ± 0.01	0.38 ± 0.01	0.35 ± 0.01	0.33 ± 0.01
100 mM (KCl) (with 15 mM buffer)	0.40 ± 0.01	0.05 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.34 ± 0.01
1 M (KCl) (with 15 mM buffer)	0.40 ± 0.1	0.060 ± 0.003	0.38 ± 0.01	0.38 ± 0.01	0.35 ± 0.01
5 mM $CaCl_2$ (with 15 mM buffer)	0.40 ± 0.01			0.38 ± 0.01	0.35 ± 0.01

Uncertainties are the standard errors from least-squares fits.
*Spectator anisotropy change from Eq. 6.
‡M state not detectable under these conditions (see text).

Δr_s is the maximum amplitude of the spectator anisotropy change, and the factor of 2 arises from the existence of two spectator proteins in each trimer. (If only one spectator reorients, its anisotropy change is twice as large.) The results of these fits for pH values over the range pH 5.0–8.0 are presented in Table 2. Fits of Eq. 6 directly to $\Delta r_s(t)$ calculated from Eq. 4 instead of Eq. 5 resulted in fitting parameters in agreement (within experimental error) with those in Table 2, but with larger uncertainties. The determination of the anisotropy of BR is complicated at pH values of 9 or greater by the increased concentration of the N intermediate (Váró and Lanyi, 1990; Ames and Mathies, 1990). Because the N intermediate absorbs at 570 nm, and its anisotropy is not known, we did not attempt to determine the anisotropy of BR at pH ≥ 9 from anisotropy decays at 570 nm on the millisecond time scale.

Our measurements of the dependence of the anisotropies of BR photointermediates on ionic strength at fixed pH were motivated by the fact that previous measurements of time-dependent properties of BR have been carried out over a range of ionic strength conditions. Hence, it is prudent to determine the relevance of this parameter to allow comparison with results from other laboratories. The dependence of anisotropies on ionic strength for BR, K, L, and M at pH 7 is summarized in Table 3. The anisotropies of the BR hole (determined at 390 μs) and of the K state are independent of ionic strength, whereas the anisotropies in L and M appear to increase slightly between 15 mM and 1 M. This result is consistent with the pH dependence in Fig. 1 *B*. Whereas the apparent pK_a above pH 7 appears to depend on ionic strength (see Fig. 1 *B*), as expected in light of the negative surface potential of the membrane (Kono et al., 1993), the magnitude of the maximum reorientation around pH 7 is not strongly affected by increased ionic strength. However, the low-ionic-strength data show decreased reorientation in the L state, and virtually no spectator reorientation. This result is consistent with the nearly constant anisotropy at 554 nm over 10 ms reported by Schulenberg et al. (1995). Apparently ions removed from the membrane at low ionic strength play a crucial role, particularly in the spectator motions. No M state was detectable under these conditions, perhaps because of incipient formation of blue membrane over the course of the experiment.

DISCUSSION

The pH of the medium has a marked effect on the anisotropy in the M intermediate and a less pronounced effect on the anisotropy of L, as shown in Fig. 1. Reorientational motions are nearly eliminated at pH < 5. The amplitude of reorientational motion reaches its greatest amplitude at a pH of 7 to 8, and is reduced for pH ≥ 9. (The M-state reorientation observed at pH 9–10 is in agreement with the reported tilt of the chromophore by 11 ± 6° in the M-state, reported by Hauss et al. (1994), based on neutron diffraction measurements of purple-membrane films at pH 9.6.) A similar pH dependence is found in the reorientation of spectator BR on the millisecond time scale. This finding is consistent with the hypothesis that spectator reorientations are triggered by the reorientation of the photoexcited protein (Wan et al., 1993). The absence of a significant dependence of anisotropy on ionic strength (Table 3) suggests that these anisotropy changes are not strongly sensitive to surface potential.

We have argued (Song et al., 1994) that whatever the specific nature of the reorientational motions in the L and M states, the fact that they are photoinduced and reversible shows that they are triggered by changes inside the protein. The fact that reorientations in L and M are maximized over the pH range 6 to 9 suggests that reorientational motions are enabled or disabled by the protonation states of key residues in the protein. These results imply that deprotonation of a group with $pK_a \approx 6$ enables reorientations, and that deprotonation of another site above pH ≈ 9 restricts reorientational motion. Interpretation of these results could follow two lines. In one, these pH-dependent changes could be attributed to heterogeneity in the initial BR populations, with pH-dependent BR populations. In the other, the pH-dependent changes would be associated with proton transfer events in the course of the BR photocycle.

Two recent observations suggest that the protonation state of a group with $pK_a \approx 9$ controls the composition of a heterogeneous initial BR population. In one, an increase in the lifetime of the retinal excited state in BR with pH was observed over the pH 9–10 range (Song et al., 1994). In the other, FTIR difference spectra showed a pH-dependent conformational change of the protein in the BR ground state (Száraz et al., 1993). Both findings argue for a pH-dependent heterogeneous BR population near pH 9. One possibility is that the group responsible for this heterogeneity is the proton release group.

Measurements of the photocurrent as a function of pH have shown that the component associated with proton release is pH dependent, with a pK_a of about 9 at salt concentrations of about 100 mM (Kono et al., 1993; Liu, 1990; the discrepancies, real or apparent, in reported measurements of the pH dependence of proton pumping are discussed by Ebrey, 1993). The proton release group, sometimes designated XH, has been identified with Arg-82 (Song et al., 1994; Balashov et al., 1993) or a tyrosine residue (Balashov et al., 1991). The pH dependence of proton pumping has also been explored by measurement of the kinetics of proton release and uptake detected by pH-sensitive dyes (Zimányi et al., 1992, 1993). Proton release in the M state, for example, is enabled by a group with $pK_a \approx 6$ (Zimányi et al., 1992), whereas the pK_a for proton uptake is ~11 (Zimányi et al., 1993). These findings have led several workers to conclude that the pK_a of XH decreases from its initial value of ~9 at the beginning of the photocycle to ~6 at the time of proton release (Kono et al., 1993; Cao et al., 1995).

In light of the pH dependence for proton release discussed above, one interpretation of the pH dependence of the M-state anisotropy is that reorientation in M is correlated with proton release on the extracellular side of the membrane. From this point of view, the changes in anisotropy at pH $\geq$ 9 can be attributed to heterogeneity in the BR ground-state population. Above this pH, proton release is lost and reorientational motions are blocked. At pH < 6, where proton release does not occur until later in the photocycle (Zimányi et al., 1992), reorientation is not triggered during the M state. Another possibility is that the reorientations observed in M are correlated with the change in electrostatic interactions in the extracellular domain that includes D85, R82, and Y57 (Cao et al., 1995).

The pH dependence of the amplitude of spectator reorientations in Fig. 2 seems to track the pH dependence of reorientations in M, as expected if reorientations in M drive a reorientation in spectator proteins. The spectator reorientation times τ_a and τ_b in Table 2 are comparable to those obtained previously by a slightly different fitting procedure (Wan et al., 1993). Interestingly, these reorientation times correspond closely with the time constants for the appearance and decay of photo-induced changes in the surface potential detected by optical probes bound to the surface (Heberle and Dencher, 1992). This suggests that the reorientation of spectator BR either responds to or causes a change in the potential experienced by a surface-bound probe. This could occur if reorientation exposes the probe to a different environment, as suggested by Heberle and Dencher (1992).

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REFERENCES

Ames, J. B., and R. A. Mathies. 1990. The role of back-reactions and proton uptake during the N→O transition in bacteriorhodopsin's photocycle: a kinetic resonance Raman study. *Biochemistry.* 29: 7181–7190.

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. Origin of the parallel photocycles of trans-bacteriorhodopsin. *Biophys. J.* 60:475–490.

Balashov, S. P., R. Govindjee, M. Kono, E. Imashova, 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.

Bashford, D., and K. Gerwert. 1992. Electrostatic calculations of the pK_a values of ionizable groups in bacteriorhodopsin. *J. Mol. Biol.* 224: 473–486.

Braiman, M. S., O. Bousché, and K. J. Rothschild. 1991. Protein dynamics in the bacteriorhodopsin photocycle: submillisecond Fourier-transform infrared spectra of the L, M, and N. *Proc. Natl. Acad. Sci. USA.* 88:2388–2392.

Braiman, M. S., K. G. Victor, and J. Lewis. 1995. Photolysis-induced pK_a increase of the chromophore counterion in bacteriorhodopsin: implications for ion transport mechanisms of retinylidene proteins. *Biophys. J.* 68:A124.

Cao, Y., L. S. Brown, J. Sasaki, A. Maeda, R. Needleman, and J. K. Lanyi. 1995. Relationship of proton release at the extracellular surface to deprotonation of the Schiff base in the bacteriorhodopsin photocycle. *Biophys. J.* 68:1518–1530.

Cao, Y., G. Váró, A. L. Klinger, D. M. Czajkowsky, M. S. Braiman, R. Needleman, and J. K. Lanyi. 1993. Proton-transfer from Asp-96 to the bacteriorhodopsin Schiff-base is caused by a decrease of the pK_a of Asp-96 which follows a protein backbone conformational change. *Biochemistry.* 32:1981–1990.

Dencher, N. A., D. Dresselhaus, G. Zaccai, and G. Büldt. 1989. Structural changes in bacteriorhodopsin during proton transfer revealed by neutron diffraction. *Proc. Natl. Acad. Sci. USA.* 86:7876–7879.

Dencher, N. A., J. Heberle, C. Bark, M. H. J. Koch, G. Rapp, D. Oesterhelt, K. Bartels, and G. Büldt. 1991. Proton translocation and conformational changes during the bacteriorhodopsin photocycle: time-resolved studies with membrane-bound optical probes and x-ray diffraction. *Photochem. Photobiol.* 54:881–887.

Der, A., S. Sander, and J. Czégé. 1988. Orientation of the chromophore plane in purple membrane. *Biophys. J.* 54:1175–1178.

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

Han, B.-G., J. Vonck, and R. M. Glaeser. 1994. The bacteriorhodopsin photocycle: direct structural study of 2 substates of the M-intermediate. *Biophys. J.* 67:1179–1186.

Hauss, T., G. Büldt, M. P. Heyn, and N. A. Dencher. 1994. Light-induced isomerization causes an increase in the chromophore tilt in the M intermediate of bacteriorhodopsin: a neutron diffraction study. *Proc. Natl. Acad. Sci. USA.* 91:11854–11858.

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

Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryomicroscopy. *J. Mol. Biol.* 213: 899–929.

Koch, M. H. J., N. A. Dencher, D. Oesterhelt, H.-J. Plohn, G. Rapp, and G. Büldt. 1991. Time-resolved x-ray diffraction study of structural changes associated with the photocycle of bacteriorhodopsin. *EMBO J.* 10: 521–526.

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.* 1183: 241–261.

Liu, S. Y. 1990. Light-induced currents from oriented purple membrane. I. Correlation of the microsecond component (B2) with the L-M photocycle transition. *Biophys. J.* 57:943–950.

Maeda, A., J. Sasaki, Y. Shichida, T. Yoshizawa, M. Chang, B. Ni, R. Needleman, and K. Lanyi. 1992. Structures of aspartic acid-96 in the L

and N intermediates of bacteriorhodopsin: analysis by Fourier transform infrared spectroscopy. *Biochemistry.* 31:4684–4690.

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–670.

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

Sasaki, J., J. K. Lanyi, R. Needleman, T. Yoshizawa, and A. Maeda. 1994. Complete identification of C=O stretching vibrational bands of protonated aspartic acid residues in the difference infrared spectra of M and N intermediates versus bacteriorhodopsin. *Biochemistry.* 33:3178–3184.

Schulenberg, P. J., W. Gärtner, and S. E. Braslavsky. 1995. Time-resolved volume changes during the bacteriorhodopsin photocycle: a photothermal beam deflection study. *J. Phys. Chem.* 99:9617–9624.

Song, L., S. L. Logunov, D. Yang, and M. A. El-Sayed. 1994. The pH dependence of the subpicosecond retinal photoisomerization process in bacteriorhodopsin: evidence for parallel photocycles. *Biophys. J.* 67: 2008–2012.

Song, Q., G. Harms, C. Z. Wan, and C. K. Johnson. 1994. Reorientations in the bacteriorhodopsin photocycle. *Biochemistry.* 33:14026–14033.

Subramaniam, S., M. Gerstein, D. Oesterhelt, and R. Henderson. 1993. Electron-diffraction analysis of structural changes in the photocycle of bacteriorhodopsin. *EMBO J.* 12:1–8.

Száraz, S., D. Oesterhelt, and P. Ormos. 1993. pH-induced structural changes in bacteriorhodopsin studied by Fourier transform infrared spectroscopy. *Biophys. J.* 67:1706–1712.

Takei, H., Y. Gat, Z. Rotyman, A. Lewis, and M. Sheves. 1994. Active-site lysine backbone undergoes conformational changes in the bacteriorhodopsin photocycle. *J. Biol. Chem.* 269:7387–3789.

Váró, G., and J. K. Lanyi. 1990. Protonation and deprotonation of the M, N, and O intermediates during the bacteriorhodopsin photocycle. *Biochemistry.* 29:6858–6865.

Wan, C. Z., J. Qian, and C. K. Johnson. 1993. Light-induced reorientation in the purple membrane. *Biophys. J.* 65:927–938.

Zimányi, L., Y. Cao, R. Needleman, M. Ottolenghi, and J. K. Lanyi. 1993. Pathway of proton uptake in the bacteriorhodopsin photocycle. *Biochemistry.* 32:7669–7678.

Zimányi, L., G. Váró, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi. 1992. Pathways of proton release in the bacteriorhodopsin photocycle. *Biochemistry.* 31:8535–8543.3