Photoreactions of bacteriorhodopsin at acid pH

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ABSTRACT It has been known that bacteriorhodopsin, the retinal protein in purple membrane which functions as a light-driven proton pump, undergoes reversible spectroscopic changes at acid pH. The absorption spectra of various bacteriorhodopsin species were estimated from measured spectra of the mixtures that form at low pH, in the presence of sulfate and chloride. The dependency of these on pH and the concentration of Cl^- fit a model in which progressive protonation of purple membrane produces "blue membrane", which will bind, with increasing affinity as the pH is lowered, chloride ions to produce "acid purple membrane." Transient spectroscopy with a multichannel analyzer identified the intermediates of the photocycles of these altered pigments, and described their kinetics. Blue membrane produced red-shifted KL-like and L-like products, but no other photointermediates, consistent with earlier suggestions. Unlike others, however, we found that acid purple membrane exhibited a very different photocycle: its first detected intermediate was not like KL in that it was much more red-shifted, and the only other intermediate detectable resembled the O species of the bacteriorhodopsin photocycle. An M-like intermediate, with a deprotonated Schiff base, was not found in either of these photocycles. There are remarkable similarities between the photoreactions of the acid forms of bacteriorhodopsin and the chloride transport system halorhodopsin, where the Schiff base deprotonation seems to be prevented by lack of suitable aspartate residues, rather than by low pH.

INTRODUCTION

Bacteriorhodopsin, located in the purple membrane, is a light-driven electrogenic proton pump in the halobacteria (Stoeckenius et al., 1978; Stoeckenius and Bogomolni, 1982; Lanyi, 1984*a*). Near neutral pH the absorption maximum of the all-*trans* form of this retinal-protein is at 568 nm, and it is a millisecond time-scale cyclic reaction (photocycle), initiated by the absorption of a photon by this chromophore, which results in the translocation of a proton across the membrane. The photocycle under these conditions includes the reactions, $BR\xrightarrow{h\nu} K \longrightarrow KL \longrightarrow L \longrightarrow M \longrightarrow N \longrightarrow O \longrightarrow BR$, where K and KL are bathoproducts in the picosecond and nanosecond time-range, respectively (e.g., Shichida et al., 1983), and M contains a deprotonated Schiff base. The thermal reactions of the photocycle are driven by the 13-*trans* to *cis* isomerization of the retinal at the initial step; in K, KL, L, M, N, but not in O and BR, the chromophore is in the 13-*cis* configuration.

At pH near 3 in the presence of buffer or salt (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Edgerton et al., 1980), or at pH near 5–6 when all cations except H^+ are removed (Kobayashi et al., 1983; Chang et al., 1985; Kimura et al., 1984), BR undergoes a red-shift to 605 nm, giving rise to the "blue membrane." There is considerable evidence to suggest that the effect of the cations is to control the surface potential, and thus the relationship of surface pH to bulk pH (Szundi and Stoeckenius, 1987, 1988; Duñach et al., 1988*a* and *b*). The purple to blue transition is thought to reflect the protonation of acidic groups in the protein and/or the lipids of the purple membrane. Unlike bacteriorhodopsin at higher pH, the altered pigment in the blue membrane does not produce an M intermediate upon illumination (Kobayashi et al., 1983; Chang et al., 1985; Chronister et al., 1986; Ohtani et al., 1986), and blue membranes do not transport protons (Drachev et al., 1978). Blue membrane at acid pH will be reconverted to purple membrane by raising the pH. Upon addition of certain anions, such as chloride, the blue membrane is converted to a purple pigment, even at low pH (Fischer and Oesterhelt, 1979; Renthal, R., personal communication). The latter is similar to, but not quite identical with, BR in its absorption maximum and resonance Raman spectrum (Smith and Mathies, 1985), and upon photoexcitation produces no M intermediate (Chronister and El-Sayed, 1987; Chronister et al., 1986). The photoreactions of the blue membrane and the "acid purple membrane" are not well understood. Some estimates of the absorption spectra of the photointermediates

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of blue membrane, and their kinetics, have been made on the basis of flash-induced difference spectra in the visible (Mowery et al., 1979; Kobayashi et al., 1983; Ohtani et al., 1986) and time-resolved resonance Raman frequencies (Chronister et al., 1986). Less is known about the photocycle of acid purple membranes.

An understanding of the mechanism of proton transport by bacteriorhodopsin requires description of the path of the transferred proton in the protein during the photocycle. Since in numerous recent reports specific suggestions are made as to which groups are protonated and deprotonated at each event in the BR photocycle, we have examined the behavior of the pigment at acid pH, where some of these groups might not be able to participate in proton transfer since the pH is below their pK_a, i.e., they are permanently protonated. Asp-85 and asp-96 may be such groups. These residues (Mogi et al., 1988; Marinetti et al., 1989; Braiman et al., 1988*b*), but not others also implicated in proton transport (e.g., asp-212, tyr-185), are absent in halorhodopsin (Blanck and Oesterhelt, 1987), where proton transfer in the absence of an external proton acceptor (Hegemann et al., 1985; Lanyi, 1986) does not take place. The results, obtained with a time-resolved multichannel analyzer in this report, confirm earlier suggestions (Kobayashi et al., 1983; Ohtani et al., 1986; Chronister et al., 1986) that blue membrane undergoes a photocycle in which the initial events are unperturbed, but the later steps are absent. This photocycle greatly resembles the photocycle of halorhodopsin in the presence of chloride. The purple pigment produced by addition of chloride at low pH, on the other hand, exhibits a photocycle in which the initial events are bypassed, but the later steps are present. This photocycle is nearly identical with the nontransporting photocycle of halorhodopsin in the presence of nitrate.

MATERIALS AND METHODS

Purple membranes were isolated from *Halobacterium halobium* strain S9 according to a standard procedure (Oesterhelt and Stoeckenius, 1974). To prevent aggregation at low pH values, the samples were included in 4-mm-thick polyacrylamide gel slabs, using a method described by Mowery et al. (1979). The 570-nm absorbance of the purple-membrane–containing gels was 0.8. Their spectra agreed, within error, with spectra of purple membrane suspensions. Before the measurements, the gel slabs were equilibrated overnight in solutions of the pH of interest. These were made by appropriately mixing 1 M Na_2SO_4 with 1 M H_2SO_4, and their pH was confirmed with a glass electrode (ORION combination pH-electrode 91-05; Orion Research, Inc., Cambridge, MA), calibrated at pH < 2 with HCl. When required, 5 M NaCl was added to obtain the stated chloride concentration.

All measurements were carried out at 22°C after light adaptation (5 min, yellow light from a 150-W tungsten–halogen source) of the bacteriorhodopsin. Absorption spectra were obtained on a spectrophotometer (model 250 UV-Vis; Shimadzu Scientific Instruments, Inc., Columbia, MD) connected to an IBM-XT computer. Transient spectra were measured as described elsewhere (Zimányi et al., 1989), with a gated optical multichannel analyzer (Princeton Instruments, Princeton, NJ). The excitation of the sample was with a nitrogen laser pumped dye laser (model LN 1000/102; Photochemical Research Associated, London, Ontario, Canada). The data analysis was with an AST 286 desktop computer, using Lotus 1-2-3 software, which provided the means for fitting all spectra simultaneously to equations containing the concentrations of BR species, protons, chloride ions, and equilibrium constants.

RESULTS

Absorption spectra of BR at low pH and in the presence of chloride

First, a detailed spectroscopic study of BR was made in the pH range of 7-0.5, at nearly constant ionic strength provided in the form of Na_2SO_4 plus H_2SO_4. The samples were then remeasured with chloride added to concentrations between 0 and 0.8 M. The spectra (not shown) revealed the three transitions observed before in similar experiments (Fischer and Oesterhelt, 1979; Mowery et al., 1979): a small blue-shift and band-broadening as the pH was lowered from 4 to 2, a large red-shift at still more acidic pH but in the absence of chloride (blue membranes), and a blue-shift upon adding chloride to the blue membranes (acid purple membranes). The spectra, which in most cases represented mixtures, were decomposed to obtain estimated spectra for the pure species (the method of calculation is described below). The four different spectral species obtained in this way are shown in Fig. 1. Conditions were found to produce three of them in almost pure states (designated here[1] as BR, BR_{blue}, and $BR_{acid\ purple}$), while the fourth, BR_{acid}, appeared always in a mixture with the others, with a maximal contribution of ~35% to the spectrum at pH 3. BR under these conditions appeared at neutral pH. Its spectrum was independent of the ion content of the solution, and the absorption maximum was at 569 nm. The absorption maximum of BR_{acid} was at 558 nm; this species was implied by earlier results (Mowery et al., 1979), but was not explicitly discussed. The spectrum of BR_{blue} was obtained at pH 1.5; as reported (Szundi and Stoeckenius, 1988), it showed no further shift at pH as low as 0.5 in the absence of chloride. This species had an absorption maximum at 603 nm, similar to the earlier described maximum of 605 nm. Large amounts of $BR_{acid\ purple}$ were produced at pH 0.5 by adding NaCl (Fischer and Oesterhelt, 1979; Renthal, R., personal communication).

The calculated spectra for BR, BR_{acid}, and BR_{blue} were obtained from spectra in the absence of chloride. As

[1] *Abbreviations used in this paper:* BR, bacteriorhodopsin species above pH 4; BR_{acid}, BR_{blue}, and $BR_{acid\ purple}$, bacteriorhodopsin species defined by their spectra in Fig. 1. BR_{blue} and $BR_{acid\ purple}$ are often referred to as blue membrane and acid purple membrane. MES, 2-(*N*-morpholino) ethanesulfonic acid.

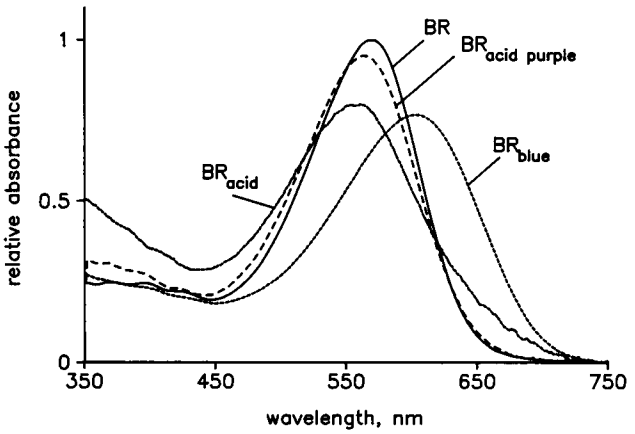


FIGURE 1 Calculated spectra for various bacteriorhodopsin species at acid pH. The measured spectra at different pH and ionic conditions were deconvoluted as described in the text. Spectra: ————, BR; ·····, BR_{acid}; ·····, BR_{blue}; ----, $BR_{acid\ purple}$. Amplitudes are shown relative to the amplitude of the absorption band of BR.

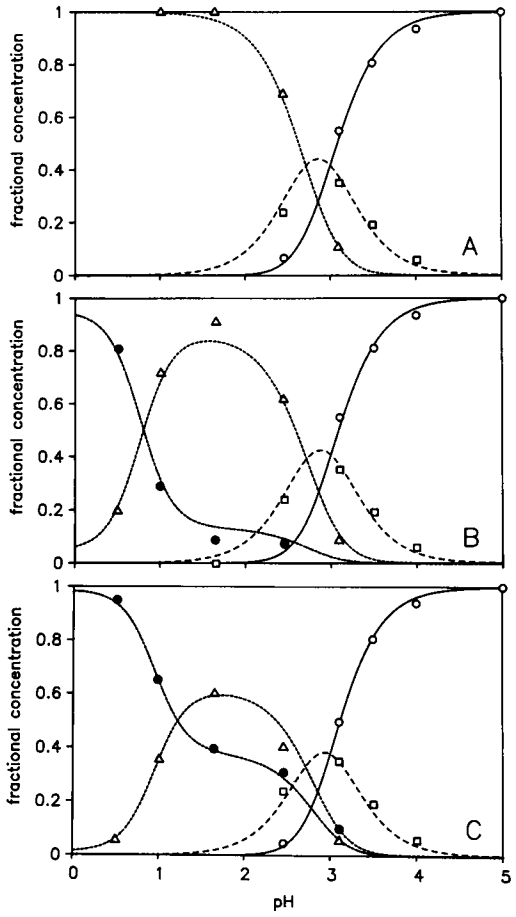


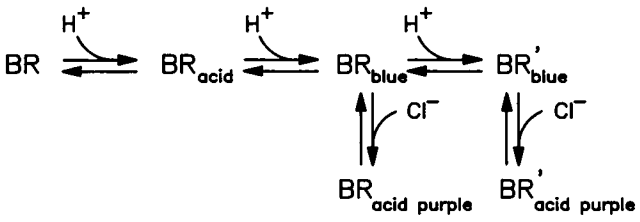
FIGURE 2 The pH- and chloride-dependent equilibria that govern the relative concentrations of the various bacteriorhodopsin species at low pH. Conditions: (*A*) 1 M Na_2SO_4 solutions mixed with 1 M Na_2SO_4 solutions to give the indicated pH; (*B*) as *A*, but with 0.2 M NaCl added; (*C*) as *A*, but with 0.8 M NaCl added. Symbols: —○—, BR; ---□---, BR_{acid}; ···△···, BR_{blue} plus BR'_{blue}; — -- —●— -- —, $BR_{acid\ purple}$ plus $BR'_{acid\ purple}$. The lines were calculated from Scheme 1.

shown in Fig. 2 *A*, the measured spectra in sulfate could be described as containing only BR at pH 5, only BR_{blue} at pH 1, but also containing BR_{acid} near pH 3. The spectrum for the latter species could be calculated by linear decomposition of the measured spectrum at pH 3.5, where only BR and BR_{acid} exist. Similarly, from the changes in the spectra at pH 0.5 with increasing chloride concentration, up to 0.8 M (cf. Fig. 2, *B* and *C*, for two of these), it was possible to obtain estimates for the spectrum of $BR_{acid\ purple}$. Extrapolation of these to infinite chloride concentration along a chloride-binding curve yielded a pure spectrum for $BR_{acid\ purple}$. At 0.8 M chloride the measured spectrum was within 5% of this calculated spectrum. Although the calculated spectrum of the $BR_{acid\ purple}$ is similar to that of the BR, it is not identical to it: the former has an absorption maximum at 563 nm, lower amplitude, and somewhat greater half-width (Fig. 1). The measured spectra could be reconstructed from linear combinations of the calculated spectra in Fig. 1, to within ± 2%.

The pH-dependent interconversions in these spectra suggest increased protonation of the pigment with decreasing pH. As noted also by Renthal (personal communication), the effect of chloride on the absorption spectra increased with decreasing pH. We suggest that the simplest model to account for this is to postulate the existence of two spectroscopically indistinguishable BR_{blue} forms, which are in pH-dependent equilibrium with one another, and which have different association constants for chloride. Hyperbolic dependence of the changes with chloride concentration argues that it is the binding of a single Cl^- that brings about the spectroscopic changes.

The lines in Fig. 2, *A–C*, were fitted to the data, to within ±5%, according to Scheme 1.

Mowery et al. (1979) found that the pH dependency of spectroscopic features indicative of these transitions were too steep to be accounted for by the protonation of single groups.



In our model also, we required more than one proton in

each step. The lines in Fig. 2, which fit all of the data, were calculated with the following parameters: BR $\longrightarrow$ BR_{acid}, pK_a = 4.2, 1.4 protons; BR_{acid} $\longrightarrow$ BR_{blue}, pK_a = 3.8, 1.4 protons; BR_{blue} $\longrightarrow$ BR'_{blue} pKa = 0.5, 2.3 protons; BR_{blue} $\longrightarrow$ $BR_{acid\ purple}$, K_{Cl} = 1.3 M, a single Cl^-; BR'_{blue} $\longrightarrow$ $BR'_{acid\ purple}$, K_{Cl} = 0.10 M, a single Cl^-. The noninteger numbers of protons suggest that the color changes in BR may be caused by cooperative processes, involving perhaps electrostatic interactions at or near the BR surface (as suggested by Szundi and Stoeckenius, 1987, 1988).

Photochemical reactions of various BR species at low pH

The photochemical reactions of the species in Fig. 1 were determined with a gated optical multichannel analyzer, described before (Zimányi et al., 1989). Spectra of the photointermediates and the kinetics of their formation and decay were calculated from difference spectra, obtained at various delay times after the flash, as in our earlier publications (Zimányi et al., 1989; Zimányi and Lanyi, 1989). The spectra of the photocycle intermediates for gel-encased BR, and their kinetics, in 0.025 M MES buffer pH 6.0 (not shown) were essentially the same as those published earlier for free, suspended purple membranes using the same instrumentation (Zimányi et al., 1989), and agreed with what has been reported by many other groups. For BR in 1 M Na_2SO_4 at pH 6.0 (Fig. 3), however, the M intermediate was followed by a species that absorbed at 550–560 nm instead of near 640 nm, i.e., the O form was replaced by an intermediate that could be identified as the N species observed by others (Kouyama et al., 1988). N probably decayed to BR via O, since a very small quantity of O could be observed in the late spectra, near the end of the decay of N (not shown in Fig. 3 *B*). Upon addition of 0.8 M NaCl to 1 M Na_2SO_4 the only observed change in the photocycle was a slowing, by about one order of magnitude, in the decay of the L form, and correspondingly in the rise of M (not shown). This had the effect that under these conditions the M state did not accumulate to the maximum fractional concentration of 1, but only to about 0.7. It should be noted that in Fig. 3, and in every case when we determined the spectrum of the M intermediate, we observed a broad band between 500 and 550 nm, in addition to the main peak near 410 nm. Such a feature can be seen in at least one reported spectrum for M (Hess and Kuschmitz, 1977).

When the pH was lowered to 3.5 in 1 M Na_2SO_4 (Fig. 4), the N form was replaced by O in the millisecond time-range. At this pH also, the spectra of the intermediates (Fig. 4 *A*) and the kinetics (Fig. 4 *B*) were similar to those observable in the complete absence of salt (not shown). An increase in the amount of O in the photocycle

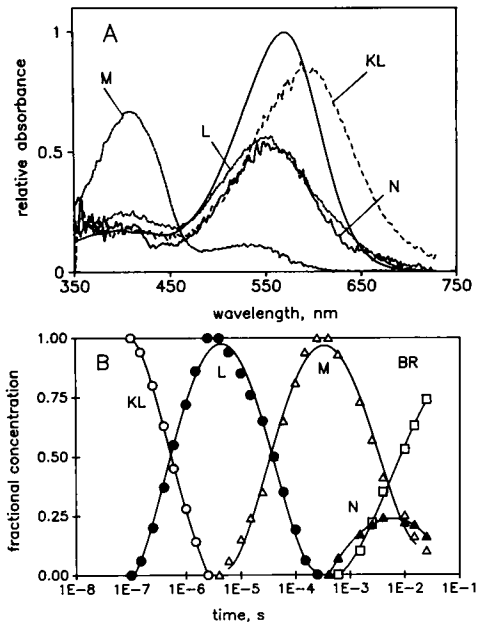


FIGURE 3 Photocycle intermediates (*A*) and their kinetics (*B*) for purple membrane in 1 M Na_2SO_4 at pH 6.0. The spectra of the intermediates are shown relative to the spectrum of the parent species. Species identified: □, ————, BR (absolute maximum at 570 nm); ○, -----, KL (absolute maximum at 593 nm); ●, ·····, L (absolute maximum at 548 nm); △, ----------, M (absolute maximum at 408 nm); ▲, -----, N (absolute maximum at 556 nm). The origin of the additional low amplitude band in the spectrum of M is not identified.

with decreasing pH was observed also by others (Li et al., 1984); its cause seems to be specific pH-dependent changes in the rate constants of the interconversions involving M, N, O, and BR (Váró, G., A. Duschl, and J.K. Lanyi, manuscript in preparation). Although at pH 3.5 in 1 M Na_2SO_4 the pigment exists as a mixture of BR and BR_{acid} (Fig. 2 *A*), no evidence of heterogeneity in the photocycle was observed (Fig. 4).

At pH 1.5, with 1 M Na_2SO_4, BR_{blue} is present virtually as the only species (Fig. 2 *A*). The photocycle under these conditions is considerably simplified. Fig. 5 contains difference spectra in the only two time-domains where transitions were observed. The difference spectra resemble, qualitatively at least, earlier point-by-point difference spectra at selected delay times for BR_{blue} (Mowery et al., 1979; Kobayashi et al., 1983; Ohtani et al., 1986). The calculated spectra of the intermediates that participate in these transitions are like the KL and L forms of BR (Fig. 6 *A*), except that the spectra of the intermediates are red-shifted by 25-30 nm, similar to BR_{blue} relative to BR. A small discrepancy in the difference spectra necessitated that a species that absorbs below 500 nm be introduced. This arises from L in the amount of ~5% (Fig. 5 *B*). The formation of L is about as rapid as at pH 7, but its decay is

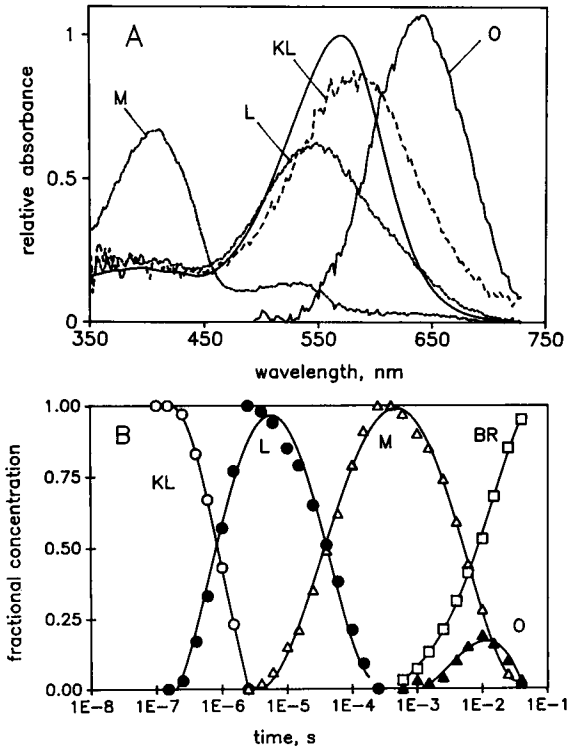


FIGURE 4 Photocycle intermediates (*A*) and their kinetics (*B*) for BR plus BR_{acid} in 1 M Na_2SO_4 at pH 3.5. The spectra of the intermediates are shown relative to the spectrum of the parent species. Species identified: □, ————, BR plus BR_{acid} (absolute maximum at 569 nm); O, -----, KL (absolute maximum at 582 nm); ●, · · · · ·, L (absolute maximum at 545 nm); △, ----------, M (absolute maximum at 405 nm); ▲, -----, O (absolute maximum at 636 nm). The origin of the additional low amplitude band in the spectrum of M is not identified.

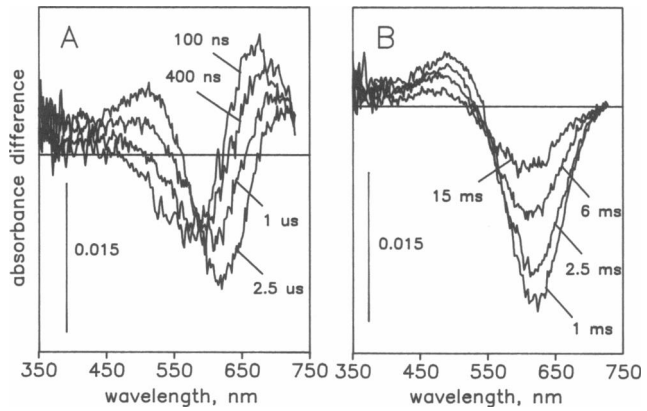


FIGURE 5 Time-resolved difference spectra for BR_{blue}. The flash-induced absorption changes are shown in the 100-ns to 2.5-μs (*A*) and the 1- to 15-ms (*B*) time ranges. Conditions, Na_2SO_4 plus H_2SO_4 (total 1 M), pH 1.5.

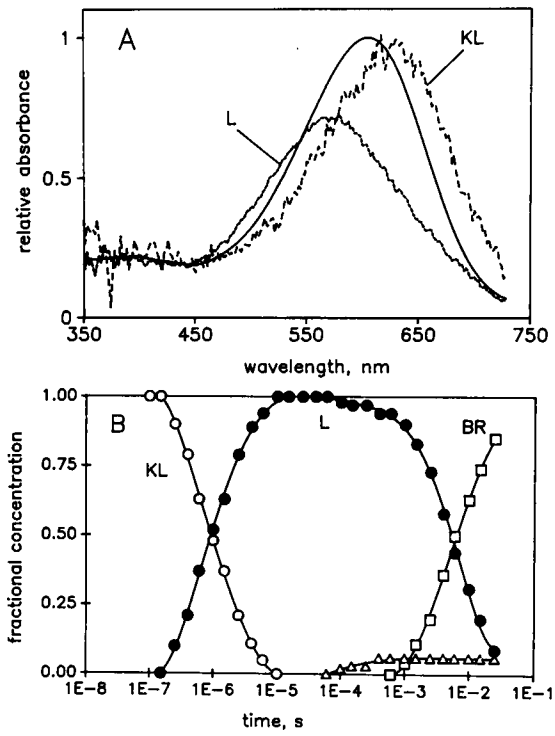


FIGURE 6 Photocycle intermediates (*A*) and their kinetics (*B*) for BR_{blue} plus BR'_{blue} in 1 M Na_2SO_4 at pH 1.5. The spectra of the intermediates are shown relative to the spectrum of the parent species. Species identified: □, ————, BR_{blue} plus BR'_{blue}(absolute maximum at 603 nm); O, -----, KL (absolute maximum at 627 nm); ●, · · · · ·, L (absolute maximum at 572 nm). Arising near 0.1–1 ms a small unidentified species was seen (△), which absorbs below 500 nm.

two decades slower; hence, unlike at pH 7, here L persists between 5 and 500 μs (Fig. 6 *B*).

At pH 0.5 plus 0.8 M NaCl, where most of the pigment is in the $BR_{acid\ purple}$ form (Fig. 2 *C*), the photocycle showed dramatic changes. Fig. 7 contains difference spectra in the two time-domains where transitions were seen. The first transition (Fig. 7 *A*), in <1 μs, is between two red-shifted intermediates with nearly the same spectra. It involves an intermediate considerably red-shifted from KL (Fig. 8 *A*); its spectrum resembles that of the KO form of halorhodopsin (Zimányi and Lanyi, 1989). This form, which we designate here also as KO, decays to a species similar to the O intermediate of BR (Fig. 8 *A*). The second transition, which takes place over a very broad time-range (Figs. 7 *B* and 8 *B*), is the return of this O to $BR_{acid\ purple}$. Similar to the results with BR_{blue}, the difference spectra contained a small discrepancy which made it necessary to postulate a species absorbing below 500 nm. This arose on the microsecond time-scale, and its decay was considerably delayed. Unlike all the other interconversions described here, the O to $BR_{acid\ purple}$ transition followed multiexponential kinetics (Fig. 8 *B*). The rea-

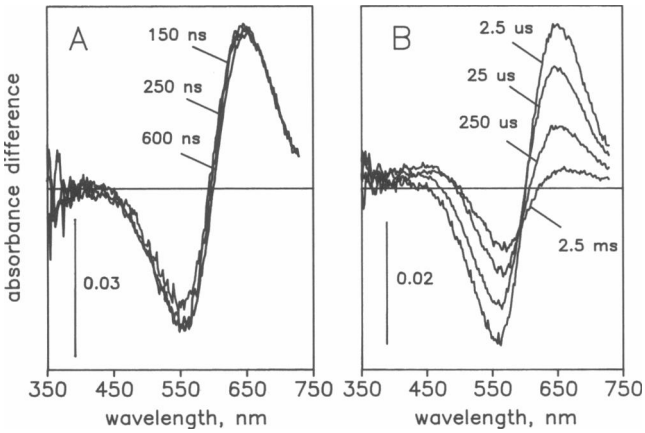


FIGURE 7 Time-resolved difference spectra for $BR_{acid\ purple}$. The flash-induced absorption changes are shown in the 150- to 600-ns (*A*) and the 2.5-μs to 2.5-ms (*B*) time ranges. Conditions, as in Fig. 5, but with 0.8 M NaCl added and at pH 0.5.

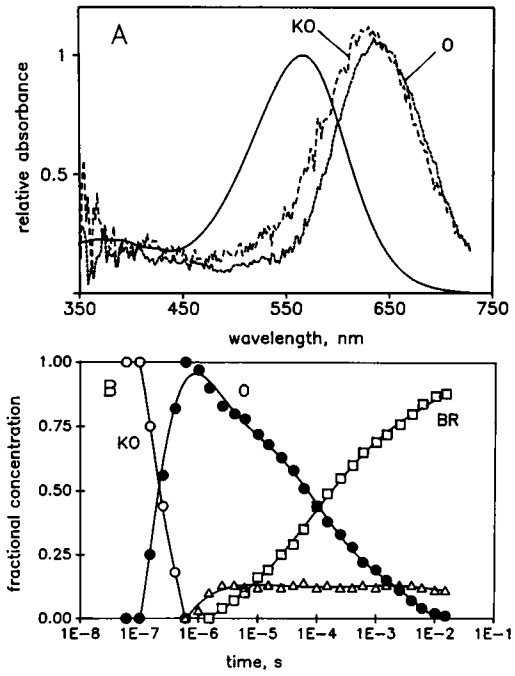


FIGURE 8 Photocycle intermediates (*A*) and their kinetics (*B*) for $BR_{acid\ purple}$ plus $BR'_{acid\ purple}$ in 0.8 M Na_2SO_4 plus 0.8 m NaCl at pH 0.5. The spectra of the intermediates are shown relative to the spectrum of the parent species. Species identified: □, ————, $BR_{acid\ purple}$ plus $BR'_{acid\ purple}$ (absolute maximum at 564 nm); ○, -----, KO (absolute maximum at 627 nm); ●, ·····, O (absolute maximum at 638 nm). Arising near 1 μs, a small unidentified species was seen (△), which absorbs below 500 nm.

sons for the latter are unclear; one possibility is that $BR_{acid\ purple}$ might be heterogeneous.

The reconstitution of the spectra of the photoproducts yielded estimates for the relative quantum yields of the photoreactions of BR and the mixtures present in the experiments in Figs. 4–8. These were 90% that of BR for the BR plus BR_{acid} mixture, 80% that of BR for the BR_{blue} species, and 50% that of BR for the $BR_{acid\ purple}$ species.

DISCUSSION

As described in previous reports, we find that bacteriorhodopsin undergoes a series of transformations as the pH is lowered. These can be described as follows: first a slightly blue-shifted (558 nm) and broadened spectral species, BR_{acid}, is produced, followed by a considerably red-shifted (603 nm) and further broadened species, BR_{blue}. Upon adding chloride, BR_{blue} undergoes a blue-shift (to 563 nm), yielding $BR_{acid\ purple}$. BR_{acid} and $BR_{acid\ purple}$ are found in mixtures, but we have been able to decompose the measured spectra to obtain estimated spectra for the pure species (Fig. 1). The calculated concentrations of these species fit a model with pH-dependent equilibria among the BR, BR_{acid}, and BR_{blue}, and a chloride-binding equilibrium between BR_{blue} and $BR_{acid\ purple}$ (Scheme 1). Mowery et al. (1979) noted that the pH dependency of the BR ↔ BR_{blue} equilibrium was too steep for a single protonation reaction, and we also assume more than one proton, binding cooperatively, in the BR ↔ BR_{acid} and BR_{acid} ↔ BR_{blue} equilibria. The binding of chloride apparently converts BR_{blue} into $BR_{acid\ purple}$ (Fischer and Oesterhelt, 1979; Renthal, personal communication). Although the chloride dependency of the BR_{blue} ↔ $BR_{acid\ purple}$ is hyperbolic (Renthal, R. personal communication), the apparent dissociation constant for chloride is pH dependent. Furthermore, the pH dependency is too steep to be produced by the binding of a single proton before the binding of the chloride. We suggest, therefore, a model in which more than one proton must be bound for eliciting chloride binding in BR_{blue}. Thus, a second BR_{blue} species is postulated, BR'_{blue}, which is spectroscopically indistinguishable from the first, but binds chloride with high affinity. The pH dependence of $BR_{acid\ purple}$ could be then fitted to the scheme, but only by including the production of $BR_{acid\ purple}$ also from BR_{blue} by chloride binding, although with much lower affinity (Fig. 2, *B* and *C*). For consistency of nomenclature, BR_{blue} gives rise to $BR_{acid\ purple}$, and BR'_{blue} gives rise to $BR'_{acid\ purple}$. However as with BR_{blue}, the two $BR_{acid\ purple}$ species are spectroscopically indistinguishable. The model we favor states, therefore, that BR_{blue} weakly binds chloride (K_{Cl} = 1.3 M), but as more protons are bound to BR_{blue}, the binding affinity increases until the anion is bound as effectively (K_{Cl} = 10

mM) as in the related retinal protein, halorhodopsin, which transports chloride rather than protons upon illumination. Since every buried, positively charged residue in halorhodopsin is also present in bacteriorhodopsin (Blanck and Oesterhelt, 1987), the ability to bind chloride must also reside in the latter protein. It seems likely that it is some, or all, of the buried acidic residues which are present in bacteriorhodopsin but not in halorhodopsin, which prevent chloride-dependent effects in this pigment, and it is their protonation at low pH which removes the masking of chloride binding site(s). These residues in bacteriorhodopsin are glu-9, asp-85, asp-96, and glu-204.

We have attempted to describe the photoreactions of the various bacteriorhodopsin species at low pH by creating conditions where the amounts of each species in the mixtures were the largest achievable. Gel-encased BR at pH 7 in 1 M Na_2SO_4 exhibited a photocycle very similar to that of free purple membrane suspensions, described by others, and ourselves, with the optical multichannel analyzer (Zimányi et al., 1989), except that the late intermediate O is replaced by N (Fig. 3). Without the Na_2SO_4, the appearance of N is generally seen at higher pH (Kouyama et al., 1988). At pH 3.5 in the absence of chloride, where BR and BR_{acid} coexist (Fig. 1), the photocycle is essentially unchanged, but instead of N, O is observed (Fig. 4). The latter is expected from the pH dependency of the appearance of O and N (Li et al., 1984; Kouyama et al., 1988). There is no evidence of photoproducts or kinetics for BR_{acid} different from those for BR; BR_{acid} is present in the amount of ~20% (Fig. 2 *A*), and differences of this magnitude would have been detectable. Thus, either the photocycle of BR_{acid} is the same as that of BR, or the quantum yield for its photoconversion is much lower than that of BR (but in that case the quantum yield relative to the photoexcitation at higher pH should have been lower than the 90% observed).

At pH 1.5, in the absence of chloride, BR_{blue} is virtually the only species present (Fig. 2 *A*). As suggested earlier (Ohtani et al., 1986), its early photointermediates resemble those of BR: The KL ⟶ L transition (Figs. 5 and 6 *B*) are both at ~1 μs, and the spectra of these species are very similar to those of BR, except for a 25–30-nm red-shift (Fig. 6 *A*). Unlike in BR, a K ⟶ KL transition was not seen in BR_{blue} (Ohtani et al., 1986). An M-like intermediate is missing (Kobayashi et al., 1983; Chang et al ., 1985; Ohtani et al., 1986; Chronister et al., 1986) (Fig. 6); instead the L ⟶ BR transition is delayed until ~5 ms. The photocycle of BR_{blue} is strikingly similar to the photocycle of halorhodopsin at high chloride concentrations (Zimányi and Lanyi, 1989), and of pharaonis halorhodopsin (Duschl, A., G. Varo, and J.K. Lanyi, manuscript in preparation). This might not be a coincidence. Other properties that BR_{blue} and halorhodopsin have in common are that neither show tyrosine deprotonation during their photocycles (Dupuis et al., 1985; Lanyi, 1984*b*; Lanyi and Vodyanoy, 1986), and upon prolonged illumination both produced quasi-stable, blue-shifted chromophores containing 9-*cis* retinal (Chang et al., 1987; Fischer et al., 1981; Maeda et al., 1980; Zimányi and Lanyi, 1987). The similarity of the photocycles of these pigments can be rationalized as follows. Deprotonation and reprotonation of the Schiff base (the formation and decay of M) in bacteriorhodopsin depend on asp-85 and asp-96, respectively (Mogi et al., 1988; Braiman et al., 1988*b*; Soppa et al., 1989; Butt et al., 1989), and these residues are missing in the two halorhodopsins (Blanck and Oesterhelt, 1987; Duschl, A., G. Varo, and J.K. Lanyi, manuscript in preparation). Tyr-185 (Braiman et al., 1988*a*) and asp-212 (Braiman et al., 1988*b*) are also involved in proton transfer in bacteriorhodopsin, but they are not the primary candidates here since proton transport was not eliminated when tyr-185 is changed to phe (Mogi et al., 1987), and replacement of asp-212 with other residues produced mixed effects (Mogi et al., 1988), suggesting gross conformational changes. The reason that BR_{blue} does not produce M might be that asp-85 is protonated in this species (Butt et al., 1989). This possibility is supported by the fact that replacement of asp-85 with asn produced a substantial red-shift in light-adapted BR, to within 10 nm of the absorption maximum of BR_{blue}, and its replacement with glu produced a BR conformation with an even greater red-shift (Mogi et al., 1988; Soppa et al., 1989). Unfortunately, the photocycle of BR with altered asp-85 has not yet been described. Permanent protonation of asp-96, instead of asp-85, would also account for lack of proton transport in BR_{blue}, but not for its red-shifted absorption maximum and the lack of M. On the other hand, still other possibilities cannot be ruled out. UV difference spectra (Renthal, R., personal communication) between BR and BR_{blue} suggest that it is tyr-185, additionally or instead of asp-85, which might be anomalously protonated in BR_{blue}. Furthermore, Gerwert et al. (1987) found no evidence in FTIR spectra that any carboxyl groups, other than those accessible to water, deprotonated when cation-free blue membrane was made alkaline.

Retinal extraction (Fischer and Oesterhelt, 1979; Mowery et al., 1979) and resonance Raman spectra (Smith and Mathies, 1985; Chronister and El-Sayed, 1987) have indicated that BR_{blue} contains a non-negligible amount of 13-*cis* retinal isomer. Other than a smaller amount of undefined spectral species which appeared between 0.1 and 1 ms (Fig. 6 *B*), we found no evidence for photoproducts from the latter. Ohtani et al. (1986) described a red-shifted intermediate attributed to the photocycle of 13-*cis* BR_{blue}, but this was observed after excitation in the UV region.

Nearly pure $BR_{acid\ purple}$ is present at pH 0.5 in the

presence of 0.8 M NaCl (Fig. 2 *C*). The photoreaction of this species produced an unusual early intermediate, with an absorption band more than 60 nm red-shifted from the maximum of $BR_{acid\ purple}$, and a very high amplitude (Fig. 8 *A*). This is unlike KL, which absorbs only 20-25 nm to red of BR, and has a lower, broader absorption band than BR (Shichida et al., 1983; Zimányi et al., 1989). This intermediate is followed by what appears to be a species quite similar to the O intermediate of BR (Figs. 7 *B* and 8 *A*). Thus, in $BR_{acid\ purple}$ the early intermediates of the BR photocycle, up to and including M, are altered or missing. The results are in disagreement with those of Chronister et al. (1986), who described resonance Raman bands upon illumination of $BR_{acid\ purple}$, which they attributed to ethylenic stretch frequencies of K- and L-like intermediates, not shifted in comparison with the analogous species from BR. It is somewhat unusual that the recovery of $BR_{acid\ purple}$ is not a single exponential process (Fig. 8 *B*). Such multiexponential kinetics, which extends the decay of the O-like intermediate to over four decades of time, was seen also in the photocycle of partially dried BR (e.g., Hess and Kuschmitz, 1977; Váró and Keszthelyi, 1983). Interestingly, the intermediates we observed for $BR_{acid\ purple}$ are virtually identical to the only species found in the secondary photocyle for halorhodopsin in the presence of nitrate (Zimányi and Lanyi, 1989). The similarity between the photoreactions of $BR_{acid\ purple}$ and one of the two forms of halorhodopsin might not be a coincidence. In halorhodopsin the secondary photocycle is thought to be the result of nitrate binding at an anomalous location (Duschl et al., manuscript in preparation); this may be the same location where BR_{blue} binds chloride to produce $BR_{acid\ purple}$. It is intriguing, therefore, that in recent results (Dér, A., R. Tóth-Boconádi, and L. Keszthelyi, personal communication), charge movements measured by photoelectric signals indicated no net transport by BR_{blue}, but the net translocation of an ion in $BR_{acid\ purple}$.

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REFERENCES

Blanck, A., and D. Oesterhelt. 1987. The halorhodopsin gene. II. Sequence, primary structure of halorhodopsin and comparison with bacteriorhodopsin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:265–273.

Braiman, M. S., T. Mogi, L. J. Stern, R. D. Hackett, B. H. Chao, and H. G. Khorana. 1988*a*. Vibrational spectroscopy of bacteriorhodopsin mutants. I. Tyrosine-185 protonates and deprotonates during the photocycle. *Proteins.* 3:219–229.

Braiman, M. S., T. Mogi, T. Marti, L. J. Stern, H. G. Khorana, and K. J. Rothschild. 1988*b*. Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartate residues 85, 96, and 212. *Biochemistry.* 27:8516–8520.

Butt, H. J., K. Fendler, E. Bamberg, J. Tittor, and D. Oesterhelt. 1989. Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1657–1663.

Chang, C.-H., J.-G. Chen, R. Govindjee, and T. Ebrey. 1985. Cation binding by bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 82:396–400.

Chang, C.-H., S.-Y. Liu, R. Jonas, and R. Govindjee. 1987. The pink membrane: the stable photoproduct of deionized purple membrane. *Biophys. J.* 52:617–623.

Chronister, E. L., and M. A. El-Sayed. 1987. Time-resolved resonance Raman spectra of the photocycle intermediates of acid and deionized bacteriorhodopsin. *Photochem. Photobiol.* 45:507–513.

Chronister, E. L., T. C. Corcoran, L. Song, and M. A. El-Sayed. 1986. On the molecular mechanisms of the Schiff base deprotonation during the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 83:8580–8584.

Drachev, L. A., A. D. Kaulen, and V. P. Skulachev. 1978. Time resolution of the intermediate steps in the bacteriorhodopsin-linked electrogenesis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 87:161–167.

Duñach, M., E. Padros, M. Seigneuret, and J. L. Rigaud. 1988*a*. On the molecular mechanism of the blue to purple transition of bacteriorhodopsin. UV-difference spectroscopy and electron spin resonance studies. *J. Biol. Chem.* 263:7555–7559.

Duñach, M., M. Seigneuret, J. L. Rigaud, and E. Padros. 1988*b*. Influence of cations on the blue to purple transition of bacteriorhodopsin. Comparison of Ca^{2+} and Hg^{2+} binding and their effect on the surface potential. *J. Biol. Chem.* 263:17378–17384.

Dupuis, P., T. C. Corcoran, and M. A. El-Sayed. 1985. Importance of bound divalent cations to the tyrosine deprotonation during the photocycle of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 82:3662–3664.

Edgerton, M. E., T. A. Moore, and C. Greenwood. 1980. Investigations into the effect of acid on the spectral and kinetic properties of purple membrane from Halobacterium halobium. *Biochem. J.* 189:413–420.

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

Fischer, U., P. Towner, and D. Oesterhelt. 1981. Light induced isomerization, at acid pH, initiates hydrolysis of bacteriorhodopsin to bacterio-opsin and 9-cis retinal. *Photochem. Photobiol.* 33:529–537.

Gerwert, K., U. M. Ganter, F. Siebert, and B. Hess. 1987. Only water-exposed carboxyl groups are protonated during the transition to the cation-free blue bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 213:39–44.

Hegemann, P., D. Oesterhelt, and M. Steiner. 1985. The photocycle of the chloride pump halorhodopsin. I. Azide catalyzed deprotonation of the chromophore is a side reaction of photocycle intermediates inactivating the pump. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2347–2350.

Hess, B., and D. Kuschmitz. 1977. The photochemical reaction of the 412 nm chromophore of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 74:20–24.

Kimura, Y., A. Ikegami, and W. Stoeckenius. 1984. Salt and pH-dependent changes of the purple membrane absorption spectrum. *Photochem. Photobiol.* 40:641–646.

Kobayashi, T., H. Ohtani, J.-I. Iwai, A. Ikegami, and H. Uchiki. 1983. Effect of pH on the photoreaction cycles of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 162:197–200.

Kouyama, T., A. Nasuda-Kouyama, A. Ikegami, M. K. Mathew, and W. Stoeckenius. 1988. Bacteriorhodopsin photoreaction: identification of a long-lived intermediate N (P,R350) at high pH and its M-like photoproduct. *Biochemistry.* 27:5855–5863.

Lanyi, J. K. 1984*a*. Bacteriorhodopsin and related light-energy converters. *In* Comparative Biochemistry: Bioenergetics. L. Ernster, editor. Elsevier Scientific Pub. Co., Amsterdam. 315–350.

Lanyi, J. K. 1984*b*. Light-dependent trans to cis isomerization of the retinal in halorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 175:337–342.

Lanyi, J. K. 1986. Mechanism of base-catalyzed Schiff-base deprotonation in halorhodopsin. *Biochemistry.* 25:6706–6711.

Lanyi, J. K., and V. Vodyanoy. 1986. Flash spectroscopic studies of the kinetics of the halorhodopsin photocycle. *Biochemistry.* 25:1465–1470.

Li, Q., R. Govindjee, and T. G. Ebrey. 1984. A correlation between proton pumping and the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 81:7079–7082.

Maeda, A., T. Iwasa, and T. Yoshizawa. 1980. Formation of 9-cis- and 11-cis-retinal pigments from bacteriorhodopsin by irradiating purple membrane in acid. *Biochemistry.* 19:3825–3831.

Marinetti, T., S. Subramaniam, T. Mogi, T. Marti, and H. G. Khorana. 1989. Replacement of aspartic residues 85, 96, 115, or 212 affects the quantum yield and kinetics of proton release and uptake by bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 86:529–533.

Mogi, T., L. J. Stern, N. R. Hackett, and H. G. Khorana. 1987. Bacteriorhodopsin mutants containing single tyrosine to phenylalanine substitutions are all active in proton translocation. *Proc. Natl. Acad. Sci. U.S.A.* 84:5595–5599.

Mogi, T., L. J. Stern, T. Marti, B. H. Chao, and H. G. Khorana. 1988. Aspartic acid substitutions affect proton translocation by bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 85:4148–4152.

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.

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of Halobacterium halobium and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–678.

Ohtani, H., T. Kobayashi, J.-I. Iwai, and A. Ikegami. 1986. Picosecond and nanosecond spectroscopies of the photochemical cycles of acidified bacteriorhodopsin. *Biochemistry.* 25:3356–3363.

Shichida, Y., S. Matuoka, Y. Hidaka, and T. Yoshizawa. 1983. Absorption spectra of intermediates of bacteriorhodopsin measured by laser photolysis at room temperatures. *Biochim. Biophys. Acta.* 723:240–246.

Smith, S. O., and R. A. Mathies. 1985. Resonance Raman spectra of the acidified and deionized forms of bacteriorhodopsin. *Biophys. J.* 47:251–254.

Soppa, J., J. Otomo, J. Straub, J. Tittor, S. Meessen, and D. Oesterhelt. 1989. Bacteriorhodopsin mutants of Halobacterium spec. GRB 2. Characterization of mutants. *J. Biol. Chem.* 264:13049–13056.

Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 51:587–616.

Stoeckenius, W., R. H. Lozier, and R. A. Bogomolni. 1978. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta.* 505:215–278.

Szundi, I., and W. Stoeckenius. 1987. Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 84:3681–3684.

Szundi, I., and W. Stoeckenius. 1988. Purple-to-blue transition of bacteriorhodopsin in a neutral lipid environment. *Biophys. J.* 54:227–232.

Váró, Gy., and L. Keszthelyi. 1983. Photoelectric signals from dried oriented purple membranes of *Halobacterium halobium*. *Biophys. J.* 43:47–51.

Zimányi, L., and J. K. Lanyi. 1987. Iso-halorhodopsin: a stable 9-cis retinal-containing photoproduct of halorhodopsin. *Biophys. J.* 52:1007–1013.

Zimányi, L., and J. K. Lanyi. 1989. Transient spectroscopy of bacterial rhodopsins with an optical multichannel analyzer. 2. Effects of anions on the halorhodopsin photocycle. *Biochemistry.* 28:5172–5178.

Zimányi, L., L. Keszthelyi, and J. K. Lanyi. 1989. Transient spectroscopy of bacterial rhodopsins with an optical multichannel analyzer. 1. Comparison of the photocycles of bacteriorhodopsin and halorhodopsin. *Biochemistry.* 28:5165–5172.