Light-induced currents from oriented purple membrane

I. Correlation of the microsecond component (B2) with the L-M photocycle transition

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ABSTRACT When irradiated, purple membrane from *Halobacterium halobium* oriented in a polyacrylamide gel produces a photocurrent. The correlation of the microsecond component (B2) of the photocurrent with the L-M optical transition was studied. It was found that the lifetimes of B2 and the L-M transition are identical over the entire pH range from 2.4 to 11.0 when measured in high salt (>5 mM $CaCl_2$ or >40 mM KCl). Changing the temperature from 10 to 35°C, or replacing the H_2O with D_2O maintains this correlation. The amplitude of B2 and the L-M transition are also correlated over the pH range where both of them can be represented as a single exponential. At high pH (>8), three exponentials were required to fit both the optical and photocurrent signals. Two of them are the previously described fast and slow components of M formation, but a new intermediate with a very fast lifetime, 0.3 μs, was observed both in absorption (λ = 410 nm) and photocurrent measurements. The lifetimes of all three were found to be pH independent. This would exclude models for the L to M portion of the photocycle that explained its complex pH-dependent behavior as being due to a single pH-dependent rate constant. The area of B2, which is proportional to the number and the distance the charge moved during the transition, is almost constant from pH 5.0 to pH 8.0. It decreases to almost zero at pH 2.0 and pH 10.6 with pKs at 2.8 and 9.1. Because B2 is thought to normally reflect proton release from the membrane, this suggests at very low and high pH the photocycle does not pump protons. The pK at high pHs for the formation of the nonpumping photocycle is probably related to the formation of a new photocycle featuring the fast rising form of M.

INTRODUCTION

Bacteriorhodopsin (bR), the only protein in the purple membrane (PM) of *Halobacterium halobium*, is a proton pump that converts light energy into a proton gradient across the membrane. bR's chromophore is a retinal covalently bound to the ε-amino group of a lysine residue via a protonated Schiff base. When bR absorbs light, it undergoes a complex photochemical cycle involving the intermediates K, L, M, N (also called R or P) and O before reverting to bR. During the L-M transition, the Schiff base is deprotonated and a proton is released on the extracellular side of the membrane. The Schiff base is reprotonated sometime between M and bR and a proton is taken up on the cytoplasmic side (for a review see Stoeckenius et al., 1979; Ebrey, 1982).

Oriented PM produces an electric current when irradiated. This photoelectric signal contains at least three major components (Drachev et al., 1978) with different lifetimes in the picosecond to millisecond range (Fig. 1): B1, a very fast component in the direction opposite to the proton movement with a rise time <100 ps (e.g., Trissl, 1985; Groma et al., 1988); B2, a microsecond range component in the same direction as the proton pump; and B3,[1] a millisecond range component in the same direction as B2. The rate of decay of each photocurrent component is equal to the lifetime of the transition between the two states producing the transition (Fahr et al., 1981; Keszthelyi and Ormos, 1983). The origin of these components is still not clear and will be partially clarified in this and the following paper. Internal charge and dipole movements of bR, proton movement, and nonproton ion movement may all contribute to these components. Because protons are transferred across the membrane during the photocycle, there must be a part of the electric signal that represents this charge movement.

Because the lifetimes of both the L-M transition and B2 component are about the same, several groups have suggested a correlation between them (e.g., Drachev et al., 1978; Keszthelyi and Ormos, 1980). However, there are discrepancies in the lifetimes of B2 reported by different labs (see e.g., Table 1 in Holz et al., 1988). Orienting the purple membrane in a low-ionic strength solution, Ormos et al. (1985) have shown a fairly good correlation for the lifetimes of the B2 photocurrent and the L-M transition from pH 5 to 8. Above pH 8, B2

[1]The terms B1 and B2 were used by Hong and Montal (1979), following the nomenclature used for the photoelectric signal from oriented rhodopsin (Cone and Pak, 1971). We have called the slowest bacteriorhodopsin component, not discussed by Hong and Montal, B3.

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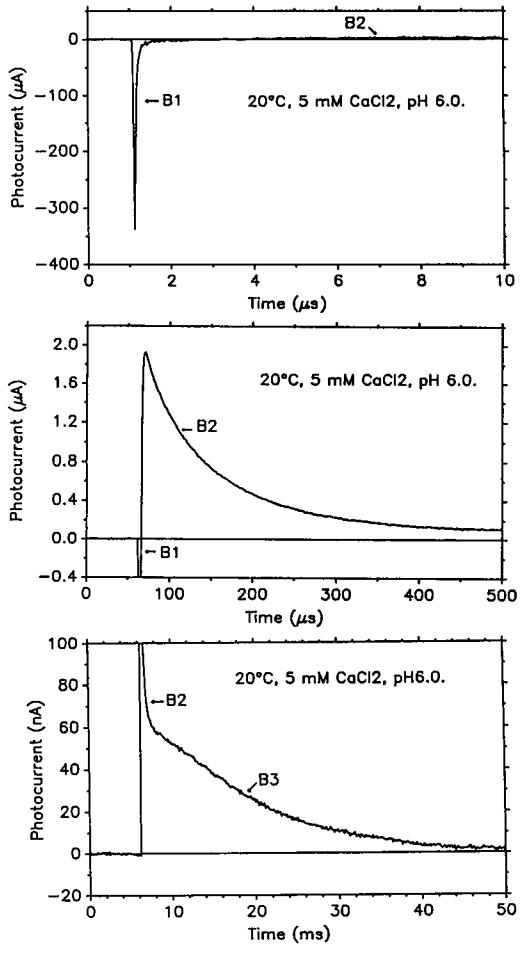

FIGURE 1 The three major photocurrent components of purple membrane, B1, B2, and B3, as seen with different time axes. The purple membrane is oriented in a polyacrylamide gel as discussed in the text.

became biphasic and the correlation disappeared. By using purple membrane oriented in a polyacrylamide gel and varying the pH with low–ionic strength buffers, Hristova et al. (1986) also showed a correlation of the lifetimes of the L-M transition and B2 component from pH 4 to 7. However, above pH 7 this correlation disappeared. Theoretically, the lifetimes of the absorption and photocurrent components should be the same if they both are due to the same transition (Fahr et al., 1981; Keszthelyi and Ormos, 1983). Here, I have studied the correlation of the optical and electrical signals using purple membrane oriented in a gel. I found that if the gels are bathed in a high–ionic strength salt solution (e.g., >5 mM $CaCl_2$ or >40 mM KCl) the correlation in lifetime of B2 and L-M transition holds for the entire pH range that the L-M transition can be observed (pH 2.4–11). This correlation also holds when the samples are measured in the D_2O, which increases the lifetime of both the L-M transition and B2 about fivefold. A correlation in amplitude holds in the pH range (pH 2.4–6.0) that both the L-M transition and B2 can be fitted with a single exponential.

The pH dependence of the L-M transition has been studied by many laboratories. Most workers agree that the L-M transition becomes faster when the pH goes above 8. Although some studies have proposed that the lifetime of L-M is pH dependent (Rosenbach et al., 1982), several groups (Hanamoto et al., 1984; Scherrer and Stoeckenius, 1985; data presented below) find that L-M transition can be fit with two pH-independent lifetimes, a ~6-μs component and a ~85-μs component at 20°C. At neutral pH, the 85-μs component dominates, and above pH 10 the 6-μs component dominates. The pK depends on the salt concentration. In this study, I will show that at high pH, there is a new 0.3-μs component that can be seen in both absorption (λ = 410 nm) and photocurrent measurements. The pH dependence of the amplitude of this new component is the same as the 6-μs component and its lifetime is pH independent too. The proportion of the 0.3- and 6-μs components, measured either optically or electrically, are constant over the pH range they can be observed.

MATERIALS AND METHODS

Purple membrane was prepared from *Halobacterium halobium* strain S-9 cells according to the method of Becher and Cassim (1975), omitting the DNase treatment.

Oriented purple membranes were immobilized in a polyacrylamide gel by the method of Dér et al. (1985), except that the final concentration of ammonium persulfate is 0.25%. The gel, which contains 28 μM bR, was first cast as a large piece of 60 × 4.9 × 50 mm with a 15 V/cm orientation voltage. This gel was washed in distilled water for at least 48 h. Then, it was cut into 6 × 4.9 × 12 mm pieces with a homemade gel slicer. Pieces that contain air bubbles or were obviously inhomogeneous were discarded. To reduce variations in PM concentration and orientation, the gels used in each group of measurements were obtained from the same large gel. They were placed into cuvettes (10 × 5 × 45 mm) and incubated in the various solutions.

The photocurrent and optical measurements were done by the same methods and equipment as described by Liu and Ebrey (1988). The excitation source was a Nd-YAG laser whose polarization was in parallel with the bR alignment; its intensity was 10 mJ.

Photocurrent measurements of gels at different pHs

The gels sliced from same large piece were incubated in 5 mM $CaCl_2$ at different pH's. To avoid distortion of the waveform, pH buffers were not used (see the next paper). The pH of each sample was measured and adjusted every 15 min for 1 h right before the measurement. Between the adjustments, the gel was air sealed. Due to residual CO_2 in the solution, the pH values between 6.8 and 8.0 may not be as reliable ($< \pm 0.3$).

Deuterated purple membrane gels

To make sure that gel was completely deuterated, two different methods were used. (*a*) The gel was placed in a cuvette and incubated in 2 ml of 99.8% D_2O containing the desired salt. The D_2O solution was changed every 2 h for a total of five times. (*b*) The gel in the cuvette containing the desired salt in H_2O was dried under vacuum (<100 millitore). Then, D_2O (99.998%, Aldrich Chemical Co., Inc., Milwaukee, WI.) was added to the cuvette. After 3 d the desiccated gel had returned to a similar size as it had initially in H_2O. The pD value of the gel was determined by adding 0.4 to the reading of a regular pH electrode (see Mikkelsen and Nielsen, 1960). Both methods gave the same results for the kinetics. But the amplitude of current signal is 20% smaller for the second method, probably due to disoreintation of the membrane by the strong mechanical forces during dehydration.

Data fitting of the kinetics traces

The number of exponentials and lifetimes of the photocurrent and absorbance traces were determined by a program using nonlinear least squares subroutine CURFIT (Bevington, 1969) written for a LSI 11-23 minicomputer (Digital Equipment Corp. Maynard, MA). At each iteration of the fitting, the program is able to present both theoretical and experimental traces on the screen, so that the quality of the fitting could be judged not only by the standard deviation but also by the residual. Because all the traces contain only 512 data points and the three components cover wide-time ranges, three traces of different full-time scales were used at each pH to accurately capture each component. Only two components plus a base line were fit in each trace and the lifetime was taken from the trace which expressed the component best. The lifetime of the fastest component was determined from the 16-μs full-scale trace. The 6-μs lifetime component was from 100-μs full-scale trace. The 85-μs component was from 500-μs full-scale trace.

RESULTS

Fig. 2 shows the lifetimes of the decay of the B2 component and the L-M transition (at 410 nm) are identical from pH 2.4 to 11 when measured in 5 mM $CaCl_2$. At this salt concentration, both B2 and the rise of M can be fit with a single exponential from pH 2.4 to 8 with the error comparable to the noise level of the signal, although two exponential fitting always gives a marginally better fit. The lifetimes of the rising phase of B2 and the corresponding small negative absorbance changes over this pH range (lifetime = 1.6 μs at 20°C) are not included in this figure because they represent the K-L transition and the corresponding charge movement (see Liu and Ebrey [1988] for details). At pH greater than ~8, both B2 and the L-M transition split into three components; their lifetimes always matched (Fig. 2). The components are designated M^{slow}, M^{fast}, and $M^{very\ fast}$. As is shown, their lifetimes are independent of the pH.

The fastest component, which has a lifetime of 0.3 μs for the photocurrent and 0.4 μs for the absorbance signal at 20°C, has not been reported previously. To show that this component is not due to an error of the fitting program, Fig. 3 *a* shows the absorbance change at 410 nm from pH 7.0 to 10.7. Clearly, a very fast component becomes apparent with increasing pH. Fitting the pH 10.7 trace with just the lifetime of M^{fast} (6 μs at 20°C, M^{slow} has essentially no amplitude under these conditions) gives a poor fit; a faster, 0.4-μs component, is required. The existence of a 0.3–0.4 μs component at high pH becomes much more obvious in the photocurrent measurements shown in Fig. 3 *b*. At neutral pH, the rising phase of B2 has a lifetime of 1.6 μs. This corresponds to a

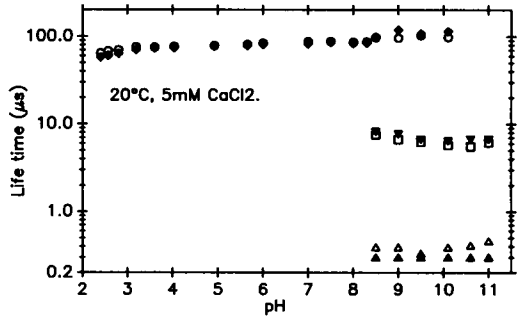

FIGURE 2 The kinetics of the L-M optical transition (measured at 410 nm) are correlated with the kinetics of B2 photocurrent component from pH 2.4 to 11.0. (*Open symbols*) Optical intermediate; (*solid symbols*) electrical intermediate.

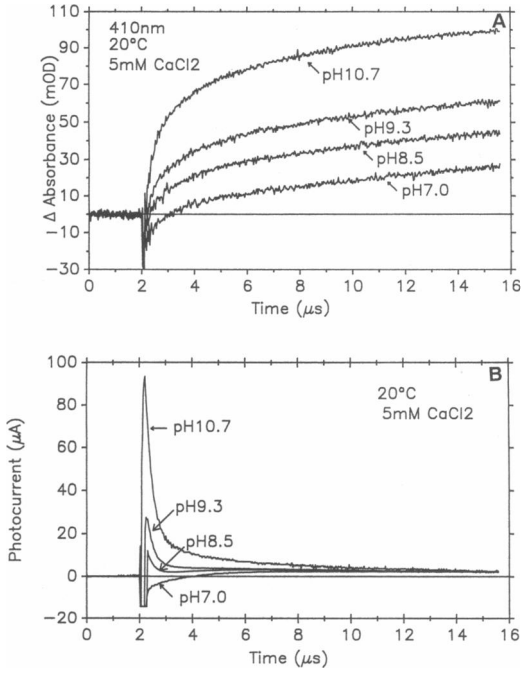

FIGURE 3 The growth in the amplitude of very fast component (lifetime = 0.3–0.4 μs) with increasing pH in the (*A*) optical absorbance and (*B*) photocurrent measurements.

combination of the K-L and L-M transitions; the K-L transition is responsible for the negative current component (see Liu and Ebrey [1988] for details). At high pH, the negative photocurrent which correlates with the K-L transition is overwhelmed by a very large positive current having two distinct decay phases, one with a lifetime of 0.3 μs and the other of 6 μs. The reason that the very fast component (0.3 μs) of the photocurrent is much better separated from the fast component (6 μs) than in the optical measurements is because the amplitude of a current component is proportional to the number of *transitions* per unit time and the distance charges move during the transition (Keszthelyi and Ormos, 1983). If the distance the charges move is about same, the amplitude of the current will be 20 times larger when its lifetime is 20 times shorter. In contrast to the electrical signal, the amplitude of an optical intermediate, say M, is proportional to the amount of pigment that is in that state. At pH 8.5, we can see a current representing the combination of the K-L, L-$M^{very\ fast}$, and L-M^{fast} transitions. The 0.3- and 6-μs components start to appear at the same pH. For the optical signal, the amplitudes of 0.4- and 6-μs components have a constant ratio of 1.38 ± 0.17 over the pH range where they could be observed. For the electrical signal, the area (the integration of current with time) of the 0.3- and 6-μs components also have a constant ratio of 0.62 ± 0.07 over the same pH range. The differences between the ratios of the optical and electrical signal is due to the different physical mechanisms generating the two signals. The pK for changing from the 85-μs component to the 0.3- and 6-μs components is 9.3 ± 0.2 at this salt concentration, 5 mM $CaCl_2$, consistent with Hanamoto et al. (1984).

The amplitudes of B2 and the L-M transition correlate over the pH range where both B2 and L-M transition can be represented as a single exponential. Fig. 4 shows the change of amplitudes of the B2 component and the L-M transition from pH 2.4 to 5.7. Upon lowering the pH, more purple membrane changes to blue membrane. Because the blue membrane does not undergo the L-M transition (Mowery et al., 1979; Kobayashi et al., 1983; Chang et al., 1985), the amplitude of M decreases. In Fig. 4 *B*, the peak amplitude of B2 decreases in the same ratio (within ±5%) as the M amplitude decreases, again indicating a close relationship between the L-M transition and the B2 component. When B2 and L-M become multiexponential at high pH, the apparent correlation in amplitude is broken because of the essential difference between the electrical (B2) and optical (L-M) transitions (see above).

Fig. 5 shows that the correlation of lifetimes of B2 and the L-M transition is maintained independent of the temperature from 10 to 35°C, as was also shown by Keszthelyi (1984). Fig. 6 shows a comparison of B2 and

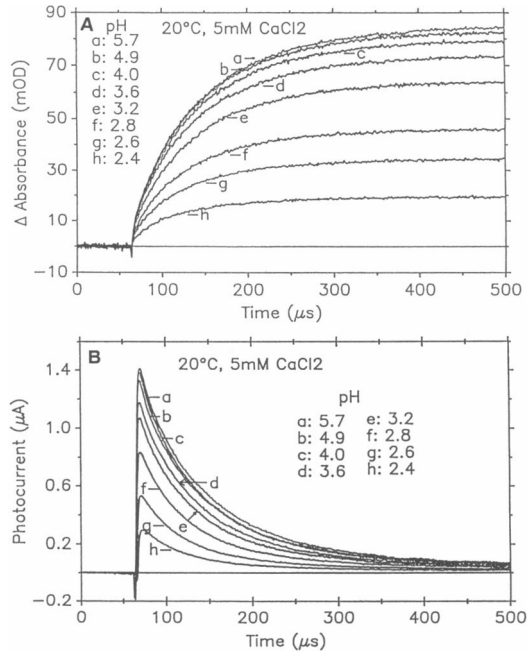

FIGURE 4 The correlation in the amplitudes from pH 2.4 to 5.7 between (*A*) the L-M optical transition at 410 nm and (*B*) the B2 photocurrent component.

the L-M transition in H_2O and D_2O. By replacing the H_2O with D_2O, the L-M transition slows down 4.63 ± 0.03–fold. This large effect suggests that the rupturing of bond involving a hydrogen, e.g., the deprotonation of the Schiff base, is the primary reaction for this transition (Katz, 1965). That the B2 component slows down to the same degree as the L-M transition when H_2O is replaced by D_2O further shows that these are closely related. This confirms the similar degree of slowing down in D_2O for both optical and electrical measurements reported by Keszthelyi and Ormos (1980). The integration of the

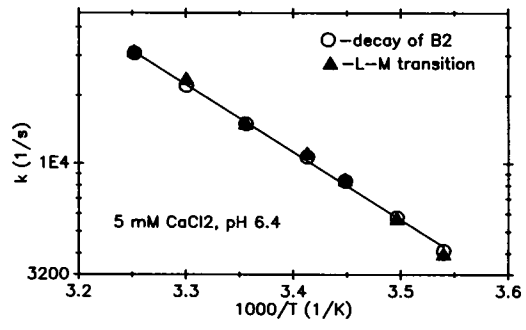

FIGURE 5 Arrhenius plots of the rate constants of L-M transition and decay of B2 from 10 to 35°C (Ea = 13.5 ± 0.2 kcal/mol, A = $1.65 \times 10^{14}\ s^{-1}$).

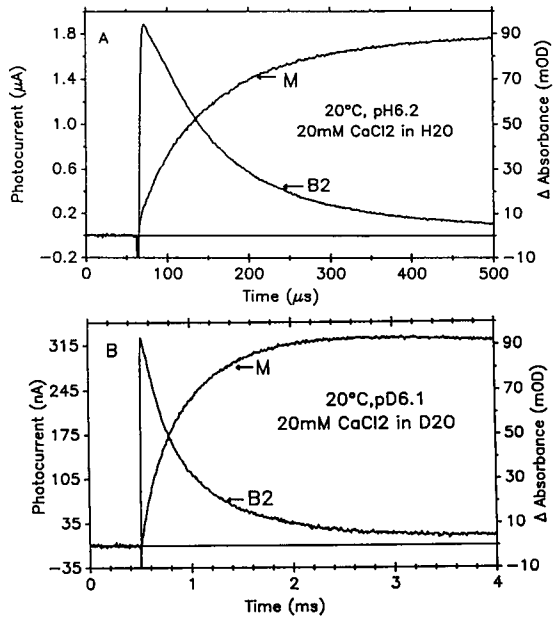

FIGURE 6 The correlation of the kinetics of the L-M transition (measured at 410 nm) and the B2 component of the photocurrent in (*A*) H_2O and (*B*) D_2O. Compared with H_2O, the lifetime of the L-M transition in D_2O is 4.6 times longer.

photocurrent over time for the B2 component is proportional to the number of bR's excited by the laser and the distance the charges move during this transition (Fahr et al., 1981; Keszthelyi and Ormos, 1983). The ratio of integrated photocurrents for H_2O and D_2O in Fig. 6 is 1.1 ± 0.1. Because the number of bR's excited are the same, this ratio indicates the distance that charges have moved during this transition is essentially the same in

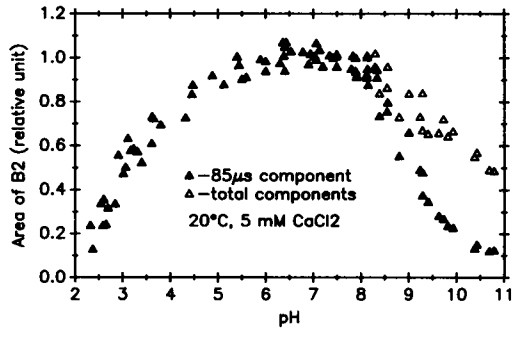

FIGURE 7 pH dependence of the area of the B2 component of the photocurrent, which is proportional to the number of charges moved and the distance they move. 5 mM $CaCl_2$, 20°C. The resolution of the integration of B2 is 32 ns and the total time integrated is 448 μs. The "area of the 85-μs component" also includes the area of "base line" of the curve fitting, which represents the small area of B3 in the same time scale.

H_2O and D_2O. The only difference is that the transition slows down.

Fig. 7 shows pH dependence of the area of B2. The area of the 85-μs component drops almost to the zero at both high and low pH with pKs at 2.8 and 9.3, respectively. With increasing pH, the total area of B2 also decreased, indicating that the very fast and fast components of B2 have less area than the slow component. The area of B2 starts to decrease at the same pH that B2 is split into three components.

In summary, all of the evidence presented above demonstrates that the B2 component of the photocurrent and the L-M transition of the optical intermediates are closely correlated both in lifetime and amplitude when they are measured in a high salt solution (>40 mM KCl or >5 mM $CaCl_2$).

DISCUSSION

Correlation between B2 and L-M transition

The lifetimes of the microsecond photocurrent component (B2) of the purple membrane reported by different laboratories are not in agreement. Moreover, the lifetimes of the B2 photocurrent and the L-M transition have not always been found to be identical. I suspect one of the reasons for this is the distortion of the waveform of photoresponse by some of the measuring systems used. Here I have used a highly accurate method for measuring the photocurrents (Liu and Ebrey, 1988). Another important reason for the inconsistency in the reported lifetimes of B2 is that the lifetime of B2 can be very sensitive to the salt concentration and the type of buffer present (see Liu et al., 1990). The lifetimes of B2 and the L-M transition are identical at all pH values only if measured at high salt (e.g., with >5 mM $CaCl_2$ or >40 mM KCl). The components of B2 reported by Ormos et al. (1985) that did not correlate with M formation at pH >8 (their components designated 4 and 5) were also observed by us when I used a low concentration of monovalent salt, 5 mM KCl (data not shown). I think these components are due to a "buffer effect" on B2 (see Liu et al., 1990) because at high pH, bicarbonate buffer is introduced into the solution from the air. A high concentration of KCl (>40 mM) can significantly reduce the effect, but divalent cations such as Ca^{++} inhibited it more efficiently.

New component at high pH

There are several studies of the L-M transition at high pH. However, the very fast component, which has a lifetime of 0.4 μs at 20°C, has not been reported previously. There are several possibilities for this. First,

many of the photoelectric measuring systems probably did not respond fast enough to resolve this component. Second, even if resolved, in the kinetic traces too few data points may have been recorded to reliably observe this component. For example, if the L-M transition was recorded with 500 μs full-time scale trace that has 1 K data points, then missing a 0.4-μs component will not introduce any visible error because this component becomes insignificant after the third data point. In fact, I was not convinced of the presence of this component until I observed the very large photocurrent signal corresponding to this intermediate. Both optical and electrical data strongly suggest the existence of a high-pH intermediate with a lifetime of 0.3–0.4 μs. The similar pH dependence of amplitudes of the 0.3–0.4-μs and 6-μs components suggest that both of them may belong to a unique photocycle that is formed at high pH; they may be related to the R intermediate (Dancsházy et al., 1986, 1988). However, further work will be necessary to test this hypothesis.

Because the amplitude of fast photocurrent component is enhanced with respect to the slower one, the photocurrent signal separates the faster components from the slow (85-μs) component more clearly than the optical measurement. From the photocurrent measurements, we can easily determine that the lifetimes of the 0.3- and 6-μs components are pH independent. This is consistent with the less complete data of Hanamoto et al. (1984) as well as Scherrer and Stoeckenius (1985). This independence excludes the possibility that there is only one photocycle over the pH 6–11 range and that the two apparent pH-dependent L-M transitions are due to the lifetime of this transition being pH dependent (Parodi et al., 1984; Ames et al., 1989). The pH-independent rate constants and pH-dependent amplitudes (see also Hanamoto et al., 1984) supports the hypothesis that bR has parallel photocycles; at high pH, bR has a second fundamentally different photocycle than that which dominates at neutral pH. The constant proportion of 0.3–0.4-μs and 6-μs components provides additional evidence to support this hypothesis. In fact, because the 0.3-μs photocurrent component has a different polarity and is faster than the 1.6-μs component corresponding to the K-L transition at normal pH, the two parallel photocycles must separate before L is formed. The most likely hypothesis is that the two photocycles occur from different forms of bR (Hanamoto et al., 1984; Dancsházy et al., 1988; Govindjee et al., 1989).

Proton pumping ability of bR at high and low pH

The area of B2 at pH 10.6 (representing almost entirely the sum of the areas of 0.3- and 6-μs components) is only 50% of the area of B2 at pH 7.0 (almost all of the area of the 85-μs component Fig. 7). Because the number of bRs excited is the same, the reduction in area of B2 could be due to two possibilities: (*a*) the number of charges each bR transfers during the fast and very fast transition is reduced; (*b*) the charge(s) moves a shorter distance during the fast and very fast transition. It seems the second possibility is most likely. Unlike the lifetime of the 85-μs component, which is very sensitive to the salt concentration and salt type (see following paper), the lifetimes of the 6- and 0.3-μs components are not sensitive to the salt type and concentration (data not shown). This suggests the 0.3- and 6-μs components do not represent a surface charge movement such as proton release. Rather they may be induced by internal charge movements associated with the high-pH photocycle, which does not pump proton(s). As will be demonstrated in the following paper, the 85-μs component represents a proton release signal. The drop of area of 85-μs component at both high and low pH suggest that purple membrane does not pump proton at both very low and very high pH. The pK's of decrease in proton release are 2.8 and 9.1, respectively. The high-pH decrease is consistent with the result of Kouyama et al. (1987). By studying the proton pumping activity at different pHs, they reported that bR does not pump protons when the pH is very high; the pK reported is ~9.5. Thus, the pK for the decrease of protons released by light is essentially identical to the pK for switching from the slow to the fast rising form of M, suggesting a common physical origin for these phenomena. This presumbly is the deprotonation of some key amino acid residue as the pH increases. This coincidence also implies that the photochemistry at neutral pH is fundamentally different from that at alkaline pH, as suggested by earlier studies.

Recently, Butt et al. (1989) have shown that a mutant of bR, in which Asp85 has been replaced by glutamic acid, has a B2 signal at neutral pH with 0.4- and 6-μs components, very similar to the B2 signal I obtained at high pH. According to Marinetti et al. (1989), replacing Asp85 with glutamic acid reduces the proton pumping ability by 68% compared with wild type bR. Thus, these photocurrent components seen at high pH in the native bR and at neutral pH in the mutant may have a common origin. One plausible suggestion is that in both cases a new pathway has been created for the proton leaving the Schiff base, a pathway that is faster (6 μs) than the normal one (85 μs), but which is available only at high pH or after Asp85 replacement. The new proton acceptor is probably a newly demonstrated group.

Ormos et al. (1985) and Hristova et al. (1986) reported that the area of the B2 photocurrent component is independent of pH from 4.5 to 10, while I found the area of B2 decreases above ~8.5 (Fig. 7). One possible reason for this

apparent discrepancy is that these workers used low ionic strength conditions, which could shift the apparent pK of the slow and fast component separation to >10. Because the exact ionic strength was not indicated, I cannot predict how far the pK will shift. Another possible reason for this discrepancy is that their comparison is based on normalizing the photocurrents according to the amplitude of the B1 component, whereas my results did not use any normalization. Because my gels are from the same large piece, all have the same concentration and degree of orientation. There are some problems in their comparing the area after normalizing by the amplitude of the B1 component. (*a*) The waveform of B1 is distorted by the recording apparatus. B1 is not a real current signal. It is related to the charging and discharging of source capacitor C_s (or C_o in their setup, because they used high impedance [very large R_o] voltage amplifier instead of a current-to-voltage converter [see Trissl et al., 1984; Liu and Ebrey, 1988 for details]). The apparent amplitude of B1 is dependent on the speed of the amplifier, the discharge of C_s, and the rising phase of B2. At high pH, the very fast component causes the apparent decay time of B1 to be <32 ns. Catching the real amplitude becomes impossible even with our fastest recording system. Therefore, the apparent amplitude at high pH will be smaller than the actual amplitude, unless the measuring system has a time resolution much better than 32 ns. (*b*) So far it has not been well established if the amplitude of B1 is really pH independent.

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