Pathways of the Rise and Decay of the M Photointermediate(s) of Bacteriorhodopsin†

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ABSTRACT: The photocycle of bacteriorhodopsin (BR) was studied at alkaline pH with a gated multichannel analyzer, in order to understand the origins of kinetic complexities in the rise and decay of the M intermediate. The results indicate that the biphasic rise and decay kinetics are unrelated to a photoreaction of the N intermediate of the BR photocycle, proposed earlier by others [Kouyama et al. (1988) *Biochemistry 27*, 5855–5863]. Rather, under conditions where N did not accumulate in appreciable amounts (high pH, low salt concentration), they were accounted for by conventional kinetic schemes. These contained reversible interconversions, either M ↔ N in one of two parallel photocycles or L ↔ M as well as M ↔ N in a single photocycle. Monomeric BR also showed these kinetic complications. Conditions were then created where N accumulated in a photo steady state (high pH, high salt concentration, background illumination). The apparent increase in the proportion of the slow M decay component by the background illumination could be quantitatively accounted for with the single photocycle model, by the mixing of the relaxation of the background light induced photo steady state with the inherent kinetics of the photocycle. Postulating a new M intermediate which is produced by the photoreaction of N was neither necessary nor warranted by the data. The difference spectra suggested instead that absorption of light by N generates only one intermediate, observable between 100 ns and 1 ms, which absorbs near 610 nm. Thus, the photoreaction of N resembles in some respects that of BR containing 13-*cis*-retinal.

Illumination of bacteriorhodopsin (BR),[1] the retinal protein of the purple membrane of halobacteria [reviewed in Stoeckenius et al. (1978), Stoeckenius and Bogomolni (1982), Lanyi (1984), and Oesterhelt and Tittor (1989)], sets off a cyclic reaction which results in the electrogenic translocation of protons from the cytoplasm into the medium. Single-turnover experiments at ambient temperature, using flash excitation and measurement of absorption transients, as well as spectroscopy of photostationary states at low temperatures, have identified distinct steps in the photochemical cycle of this pigment, which begin with the formation of the transient state named J and lead via intermediates identified by their absorption spectra, named K, KL, L, M, N, and O, back to BR in tens of milliseconds. Of the photocycle steps, the rise and decay of M assume special significance, because in the M state the Schiff base of the retinal is deprotonated, and the removal and subsequent replacement of this proton are clearly the key events in the ion translocation mechanism.

Identification of the specific residues in the deprotonation and reprotonation of the Schiff base has now begun through studies of BR containing specific residue replacements produced by site-specific mutagenesis (Marinetti et al., 1989; Braiman et al., 1988) or natural mutations (Soppa et al., 1989). In particular, evidence exists that Asp-85 might function as the proton acceptor during the deprotonation of the Schiff base (Mogi et al., 1988; Braiman et al., 1988; Butt et al., 1989; Soppa et al., 1989), while Asp-96, which is in the protonated state at the beginning of the photocycle, is implicated as the residue which later reprotonates it (Braiman et al., 1988; Mogi et al., 1988; Soppa et al., 1989; Holz et al., 1989; Butt et al., 1989). Although replacement of each of the tyrosines individually did not eliminate proton transfer (Mogi et al., 1987), Tyr-185 was reported to change its protonation state during the photocycle, and was therefore also assigned a proton transfer role (Ahl et al., 1988; Braiman et al., 1988).

Detailed interpretations of changes in the photoreaction of BR upon residue replacements are made difficult, however, by the well-documented complexities and ambiguities in the kinetics of the photocycle of wild-type BR. Numerous observations have indicated that in the late time domain several intermediates (M, N, and O) accumulate simultaneously [e.g., see Lozier et al. (1975, 1978), Nagle et al. (1982), and Beach and Fager (1985)]. Proposed explanations have included the following: (a) the rate constants of some of the interconversions are not sufficiently separated from one another (Gillbro, 1978; Nagle et al., 1982); (b) back-reactions lead to transient equilibria between some of the intermediates (Parodi et al., 1984; Chernavskii et al., 1989); and (c) branching reactions produce parallel pathways (Sherman et al., 1976, 1979; Lozier et al., 1978; Beach & Fager, 1985). We have attempted recently,[2] with optical multichannel spectroscopy, to dissect the reaction sequence at pH 4–7 and proposed a scheme for that part of the photocycle which involves M, N, O, and BR. The scheme links these species in the linear reaction sequence M → N ↔ O. The results suggested that the recovery of BR occurs either directly from N (at lower pH, where N → O is a branching reaction) or from O (at higher pH, where O is in the pathway to BR). The kinetics were consistent with a recent study of the kinetics after thermal perturbation

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[1] Abbreviations: BR, bacteriorhodopsin; BisTris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

[2] G. Váró, A. Duschl, and J. K. Lanyi, unpublished results.

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(Chernavskii et al., 1989), which established that a rapid equilibrium must exist between N and O before relaxation to BR.

Another complication long recognized is the occurrence of several kinetically distinguishable apparent M species, which are most evident at pH above 7 (Hess & Kuschmitz, 1977; Ohno et al., 1981), and particularly at high pH *plus* several molar salt concentration (Ohno et al., 1981; Kouyama et al., 1988). Thus, both the rise and decay of M could be fitted with two (or, according to some authors, three or more) exponentials. The pH dependencies of these tend to associate (Ormos et al., 1985) the more rapid rise of M with the slower decay, and vice versa.[3] El-Sayed and co-workers have proposed from the rise kinetics that there are two M species, M^f and M^s, which originate from two BR species, differing in the dissociation state of a tyrosine (Hanamoto et al., 1984). Results of Ebrey and co-workers suggested a similar model (Dancsházy et al., 1988). However, Kouyama et al. (1988) recently proposed that M^s is a photoproduct not of BR but of the photointermediate N, which accumulates during the photoexcitation of BR because of its greatly lengthened lifetime at alkaline pH and high salt concentrations. Thus, M^s was suggested to arise by the two-photon reaction, BR $\xrightarrow{h\nu}$ N $\xrightarrow{h\nu}$ M^s. However, Ames et al. (1989) stated that M^s could be produced under conditions where significant accumulation of N was not expected. While Kouyama et al. (1988) had allowed for this with a postulated limited thermal equilibration of BR with N, such an equilibration would have to had to produce large-scale replacement of BR with N to account for the results under highly alkaline conditions, in resemblance to the model of El-Sayed and Ebrey.

We have undertaken a study of the photoreactions of BR at alkaline pH, under conditions where N does not accumulate (low-salt concentration) and under other conditions when it does (high salt concentration and background illumination). The results indicate that the biphasic kinetics for M are produced independently from N. The photoreaction of N is suggested to produce not M, but a red-shifted species instead, somewhat similarly to the photocycle of 13-*cis*-BR (Dencher et al., 1976; Sperling et al., 1977; Kalisky et al., 1977).

Materials and Methods

Purple membranes were isolated from *Halobacterium halobium* strain S9 according to Oesterhelt and Stoeckenius (1974). As described under Results, in some experiments the purple membranes were suspended in buffer, while in others they were included in 4-mm-thick polyacrylamide gel slabs, using a method described by Mowery et al. (1979). In either case, the 570-nm absorbance (4-mm path length) was about 0.8. The spectra of the gel-encased samples agreed, within error, with spectra of purple membrane suspensions. Before the measurements, the gel slabs were equilibrated overnight in solutions of the pH and salt content of interest. Monomeric BR was prepared from purple membranes by solubilization with Triton X-100, as described by Dencher and Heyn, (1978); the product was confirmed as monomeric by lack of sedimentation and a shift of its absorption maximum from 568 to 552 nm.

All spectroscopic measurements were carried out at room temperature, after light adaptation of the samples. Stationary, nonilluminated spectra were measured with a Shimadzu UV-250 double monochromator spectrophotometer. Transient spectra were measured with a gated optical multichannel analyzer (Princeton Instruments, Princeton, NJ) and fitted to kinetic models, as described elsewhere (Zimányi et al., 1989; Zimányi & Lanyi, 1989). Readout was either at a frequency of 1–4 Hz and 20–50-ms exposure to the measuring light (in experiments where no steady-state accumulation of N occurred, i.e., without KCl) or at 0.25 Hz and 50-ms exposure to the measuring light (where N accumulation was induced, i.e., with KCl). The measuring window was 40 ns between 100 and 400 ns, 150 ns between 600 ns and 2.5 μs, and 1.2 μs at longer times. The excitation of the sample was with a subnanosecond pulse from 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 123 software.

Where indicated, continuous background illumination at 632.8 nm from a 1-mW HeNe laser (Uniphase Model 1301) was applied during the optical multichannel analyzer measurements. This background light was directed to the sample so as to overlap the volume illuminated by the measuring and actinic beams, while avoiding the collection of measurable intensities on the array.

Results

Photoreactions of BR at Alkaline pH in the Absence of Added Salt. Purple membranes were suspended in buffers containing 25 mM BisTris propane *plus* 25 mM CAPS, pH between 7 and 10.5, light-adapted, and the absorption spectra of the samples were measured within a few minutes. The spectra indicated that the absorption maximum remained unchanged, within ±1 nm, in the pH region examined but a small absorbance decrease at 570 nm (a few percent), as well as a small absorbance increase at wavelengths below 400 nm, occurred at the higher pH, particularly at pH 10.5 (not shown). The latter suggested limited destruction of the chromophore. Since the absorption band of N was estimated to be 8–15 nm blue-shifted and its amplitude significantly lowered relative to those of BR (Lozier et al., 1975; Kouyama et al., 1988),[2] little or no N could have accumulated under these conditions in the dark.

The same samples were used to determine flash-induced absorption transients between 350 and 750 nm with a gated multichannel analyzer, as described before (Zimányi et al., 1989). Care was taken to keep the frequency of flash and readout repetitions low enough to prevent temporal overlap between successive photocycles. In these measurements, spectra after flash excitation were recorded alternating with spectra without flash. Spectra averaged in the latter files were unchanged throughout the entire series of flash regimes (not shown) and were virtually identical with the spectra determined before the illumination (Figure 1A). Thus, under these conditions, neither the measuring light nor the flash excitation caused significant steady-state accumulation of N.

Figure 2 contains representative difference spectra at pH 10.5, measured at various delay times after photoexcitation. Even at first inspection, the spectra revealed differences from those obtained earlier at lower pH (Zimányi et al., 1989). At short delay times (Figure 2A), a decreasing absorption band near 640 nm (the decay of KL) and increasing absorption near 410 nm (the rise of M), both occurred already at delay times as early as 400 ns. Unlike at lower pH, the absence of a clear isosbestic point near 450 nm suggests that more than two species (i.e., KL, L, and M, cf. below) coexist at these times. The events at longer delay times (Figure 2B) were somewhat simpler, but the decrease of absorption near 410 nm (the decay of M) did not produce a clear isosbestic point either (because

[3] Confusingly, in some publications "slow M" refers to the slowly rising M, while in others to the slowly decaying M.

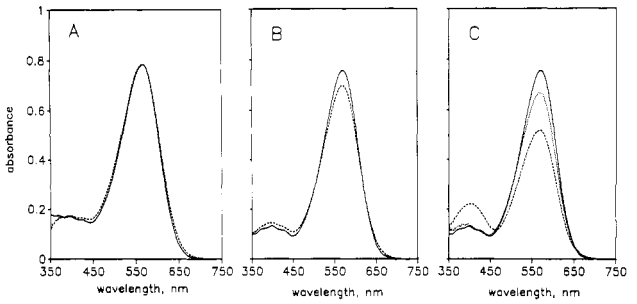

FIGURE 1: Absorption spectra of BR in the absence of actinic light, and during the optical multichannel measurements. (A) BR in suspension at pH 10.5, no added KCl. Spectrum in the absence of actinic light (measured with a Shimadzu spectrophotometer) (—); spectrum from measurements interleaved between flash excitations in the optical multichannel analyzer (as explained in the text) (---). (B) BR encased in the polyacrylamide gel at pH 10.5, with 3 M KCl. Spectrum in the absence of actinic light (measured with a Shimadzu spectrophotometer) (—); spectrum from measurements interleaved between flash excitations in the optical multichannel analyzer, no HeNe laser background light (as explained in the text) (---). (C) BR encased in the polyacrylamide gel at pH 10.5, with 3 M KCl. Spectrum in the absence of actinic light (measured with a Shimadzu spectrophotometer) (—); spectrum from measurements interleaved between flash excitations in the optical multichannel analyzer, in the presence of HeNe laser background light (as explained in the text) (---). Spectrum from which the contribution of M is eliminated by adding a BR *minus* M difference spectrum (as explained in the text) (•••).

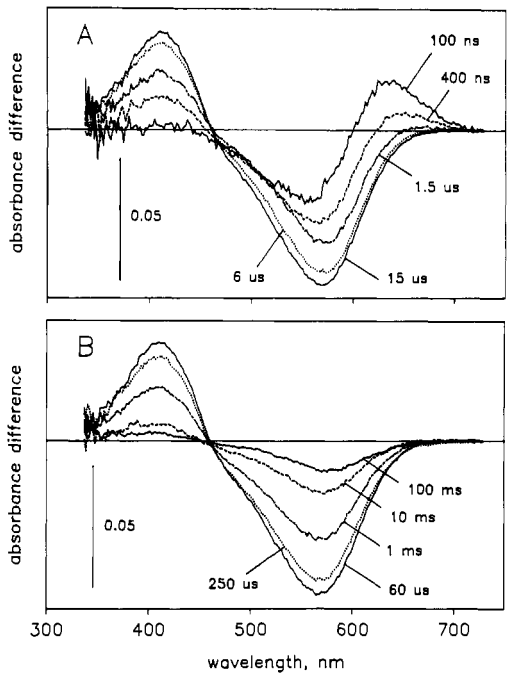

FIGURE 2: Representative time-resolved difference spectra for BR at pH 10.5, without added KCl, at early (A) and late (B) times after photoexcitation. Delay times in (A): 100 ns (—); 400 ns (---); 1.5 μs (----); 6 μs (•••); 15 μs (—). Delay times in (B); 60 μs (—); 250 μs (•••); 1 ms (----); 10 ms (---); 100 ms (—). Conditions as in Figure 1A.

N is produced before BR recovers, cf. below), and the recovery of the main depletion band (i.e., that of BR) is unusual in that it occurred over longer than 4 decades of time. Another anomaly is that the difference spectrum remained virtually unchanged between 15 μs (Figure 2A) and 60 μs (Figure 2B), indicating that at pH 10.5 the rise and decay of M are temporally considerably separated.

The fractional concentrations of intermediates L, M, N, and O, as functions of time after photoexcitation, were estimated from these difference spectra, using criteria described before

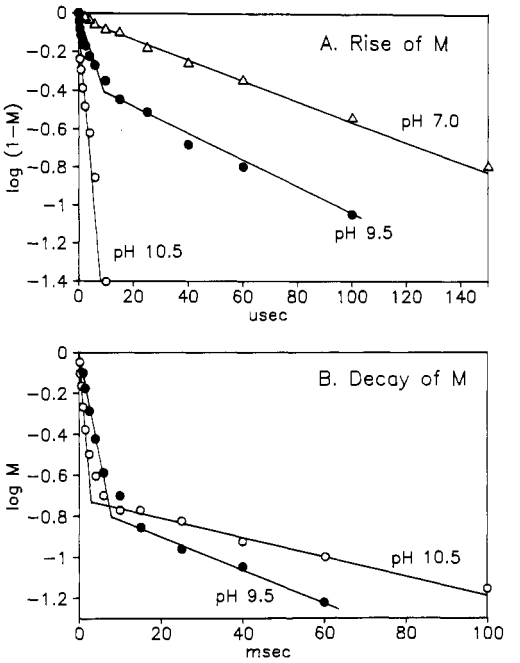

FIGURE 3: Kinetics of the rise (A) and decay (B) of M at various pHs, without added KCl. Points obtained from difference spectra, such as in Figure 2; lines were drawn by hand. pH 7.0 (△); pH 9.5 (●); pH 10.5 (○).

(Zimányi et al., 1989). Figure 3A,B shows rise and decay kinetics for M at pH 7, 9.5, and 10.5. Both rise and decay kinetics are complex, but in different ways. The rise kinetics (Figure 3A) appear to be composed of two or more exponentials; as usual in such plots, the early time points tend to run together (the lines in Figure 3 were hand-drawn and not intended to suggest an interpretation). The time constant of the slower process, at least, did not appear to be dependent on the pH, but its amplitude increased relative to the faster process, as found in an earlier report (Hanamoto et al., 1984). The decay kinetics (Figure 3B) are also accounted for by two exponentials, but here the time constants varied much more with pH than the amplitudes. Thus, any model proposed for M would have to explain the kinetic complexities of its rise and decay (Figure 3) by means which can account for their different behavior.

The fractional concentrations of the measured intermediates are shown in Figure 4, at pH 7, 9.5, and 10.5, on a 7-decade logarithmic time scale. In this analysis, the amounts of N and O were combined, since at all but the lowest pH used here, very little O accumulated during the photocycle. The data (points in Figure 4) indicate that the rise of M, noticeably biphasic at higher pH in this graph also, occurred at significantly earlier times with increasing pH, causing the transient accumulation of L to decrease. Conversely, the faster component of the biphasic decay of M became faster, and the slower component became slower, with increasing pH. Since the recovery of BR slowed down markedly with increasing pH, the transient accumulation of N (plus O) was increased. The logarithmic time scale has the advantage over linear time scales that kinetic models can be fitted to the entire time domain simultaneously, and with equal weight given to all data points. The data in Figure 4 were consistent with two different representative models, as shown in Figure 5. The first, in Figure 5A, contains two BR species in pH-dependent equilibrium, which produce photocycles I and II, respectively. The second, in Figure 5B, contains, on the other hand, a single photocycle, but with a reversible reaction giving rise to M. The essential difference between these schemes is how they account for the

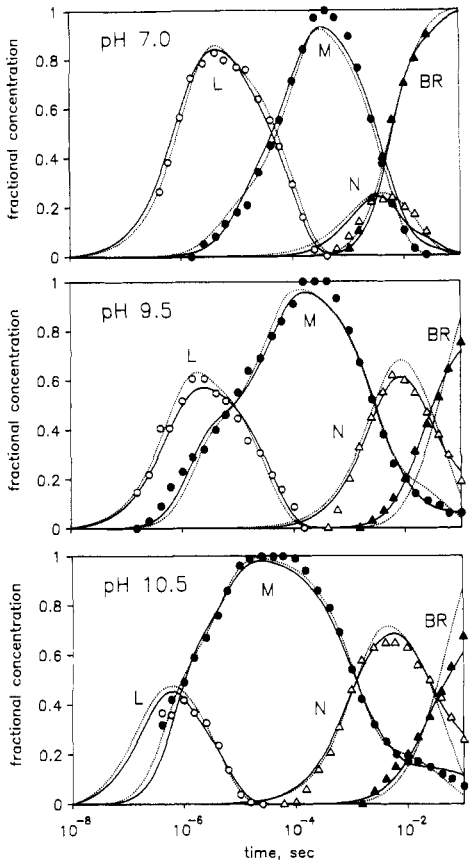

FIGURE 4: Kinetics of the intermediates L, M, N *plus* O, and BR, at pH 7.0, 9.5, and 10.5, in the absence of added KCl. Points were calculated from difference spectra, such as in Figure 2. The solid lines are from fitting the kinetic model shown in Figure 5A to the data, while the dotted lines represent the best fit for the model in Figure 5B. L (○); M (●); N (△); BR (▲). At pH 7, where a small amount of O was also seen, it was included with N.

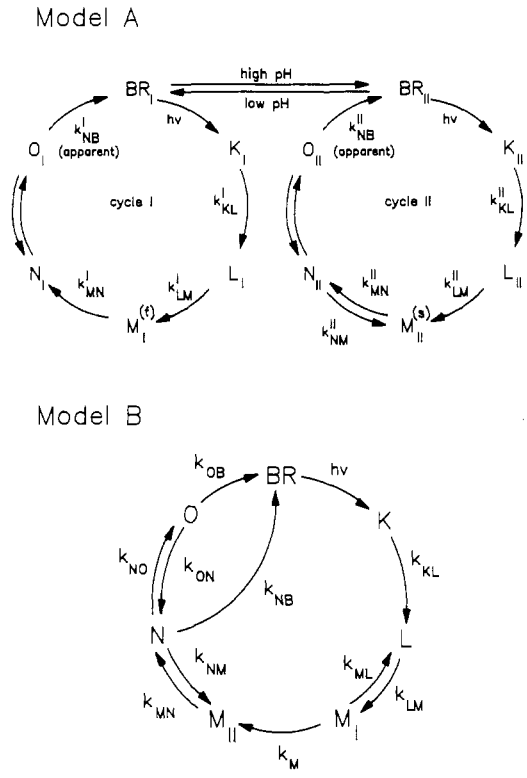

FIGURE 5: Models which account for the kinetics of the BR photocycle at low salt concentrations. The rationale for these and the reasons for rejecting other models are given in the text. In both models, the anomalous decay of M is attributed to a reversible N ↔ M step. The models differ in how they account for the anomalous rise kinetics for M. (A) Parallel photocycles (or branching before L, not shown). The rate constants for the two terminal steps are shown as apparent k_{NB}^{I} and k_{NB}^{II}, because for the sake of simplicity, steps involving O, which accumulates only slightly above pH 7 under these conditions, were ignored. (B) Single photocycle with a reversible L ↔ M step and two M forms. In this model, the steps between M and BR (footnote 2) are all given.

deviation of the rise of M from a single exponential. We considered that the biphasic rise of M is described by the general equation $[M]_t = f[\exp(-k_1 t)] + (1-f)[\exp(-k_2 t)]$. Such rise kinetics could originate either from two parallel pathways, each producing M, or from the reaction sequence L ↔ M → N. In the former, the preexponential terms represent the fractions of the two pathways, and are therefore independent of k_1 and k_2. In the latter, the preexponential terms are determined by k_1 and k_2 [for these equations in another context, cf. Duschl et al. (1988)]. We found that both models could be reasonably well fitted to the data for the rise of M in Figure 4.

The lines in Figure 4 were generated with models A (solid lines) and B (dotted lines) in Figure 5, with rate constants which, by trial and error, gave the best possible fit. Finding these rate constants was made easier by the fact that the kinetics before the maximal amount of M was reached could be fitted independently from the kinetics at later times. The equations used are given in the Appendix. Although model A is essentially the same scheme as suggested earlier by Hanamoto et al. (1984), the data in Figure 4 required that, unlike these authors, we not only have the proportions of the two pathways but also have each of the time constants in the rise of M change with pH. Furthermore, a simple model containing two parallel photocycles would predict that the proportions of the two components are the same in the rise and decay kinetics. According to our data, this is not so. Although the fractions of the faster rise component and the slower decay component both increased with pH, the latter increased much less than the former (Figure 3). This kind of result would be consistent with two parallel photocycles only if either the two M species (I and II) were interconvertible or one of the M species decayed via the fast reversible reaction M ↔ N, which would deplete part of it rapidly while prolonging the lifetime of the remainder. The first alternative was not consistent with the data, since if the required $M_{II} \rightarrow M_I$ reaction were rapid, the slower component of the M decay would never be observed, and if it were slow, the fractions of the two M decay components would remain unchanged. The second alternative, on the other hand, accounted for the results and was consistent with the fact that at high pH the accumulation of N had increased (Figure 4). The scheme in Figure 5A therefore contains this feature. In this simplified analysis, the recovery of BR from the two N species was taken as two single processes, with rate constants k_{NB}^{I} and k_{NB}^{II}. This is justified by data in this as well as in an earlier study,[2] which show that at pH >7 BR is regenerated without significant accumulation of O. Although the version of model A in Figure 5A includes two initial BR species, the data do not decide between this possibility and another, where the photocycle splits into two branches at or before the rise of L. For example, from similar data, Maurer et al. (1987) suggested branching in the photocycle at the K → L reaction.

The second model (Figure 5B) contains one photocycle, and the biphasic kinetics for the rise of M are generated instead by a reversible L ↔ M reaction. In such a scheme, decay of L to zero during the rise of M cannot occur[4] if the forward

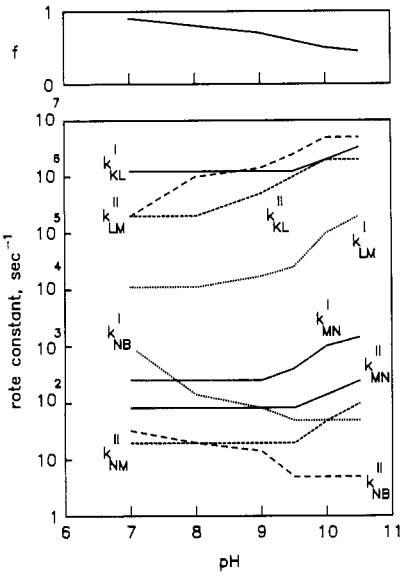

FIGURE 6: pH dependence of the fraction of photocycle II (f) and the rate constants for model A in Figure 5. f (—); k_{KL}^{I} (—); k_{LM}^{II} (--); k_{KL}^{II} (---); k_{LM}^{I} (···); k_{MN}^{I} (-·-); k_{NB}^{I} (··Å·); k_{MN}^{II} (—); k_{NM}^{II} (---); k_{NB}^{II} (--).

and reverse rate constants are of the same order of magnitude (as required to fit the data). For this reason, in this kind of a model the reversible L ↔ M_I reaction must be followed by an irreversible reaction $M_I \rightarrow M_{II}$, where the two M species are, to a first approximation at least, spectroscopically indistinguishable. Fitting of model B to the data (not shown) required that the latter reaction be so fast that when the decay of M begins, M consists virtually entirely of M_{II}. A biphasic decay for M was then generated by the reaction sequence M_{II} ↔ N (and O) → BR. The independent origins of the kinetic complexities in the rise and decay of M ensured that the model would fit the observed differences in these kinetics (Figures 3 and 4). Here we used the rate constants of all identified steps in the fitting, as indicated in Figure 5B, utilizing the general features of a pathway for the recovery of BR, derived earlier,[2] but this is not an essential difference between the two models.

Because of earlier suggestions (Ohno et al., 1981; Korenstein et al., 1979) that the biphasic M decay kinetics might be caused by cooperativity in the BR trimer, we repeated the experiments described in Figures 2–4 with monomeric BR. With this sample at pH 7, the kinetics of M rise and decay were comparable to those obtained at pH 10 with purple membranes (not shown). Although the reason for the different pH dependence of monomeric BR is not yet clear, the results clearly demonstrate that the complex M kinetics do not require that BR be in a crystalline array. This was suggested also by earlier results of Xie et al. (1987), and our own results (not shown), which indicate that the biphasic rise and decay kinetics of M are unchanged at different flash intensities (which excited between 2 and 7% of the BR, at pH 9.5).

The solid lines shown in Figure 4 were calculated by choosing appropriate values for the rate constants in model

[4] Whether the concentration of L really reaches zero at the time when maximal M is produced is made somewhat problematical by the fact that the derived spectrum of M often contains a so-far unexplained shoulder at 500–550 nm [e.g., see Hess and Kuschmitz (1977), Zimányi et al. (1989), and footnote 2]. This is very likely related to the long-lived, second L species suggested on the basis of resonance Raman spectra (Alshuth & Stockburger, 1986). However, we found that the shoulder does not appear in M spectra measured at pH >9.5 (cf., for example, Figure 9), and our analysis of the photocycle at high pH, based on the kinetics of L as drawn in Figure 4, seems justified.

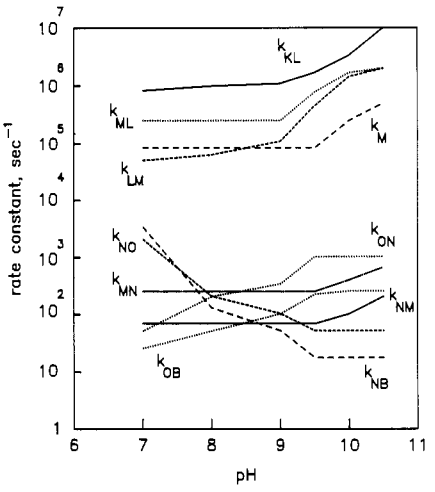

FIGURE 7: pH dependence of the rate constants for model B in Figure 5. k_{KL} (—); k_{ML} (···); k_{LM} (---); k_M (--); k_{NO} (---); k_{ON} (···); k_{MN} (-·-); k_{NM} (—); k_{OB} (···); k_{NB} (--).

A (Figure 5A) and the fraction of BR which entered photocycles I and II at the selected pH. These parameters, and additional ones determined at intermediate pH, are shown in Figure 6 as functions of pH. The rate constants show either a distinct increase at pH >9, or in two cases, for the two N → BR reactions, a decrease with pH. Unexpectedly, both forward and reverse rate constants of the reaction N_{II} ↔ M_{II}, i.e., k_{NM}^{II} and k_{MN}^{II}, increased with pH. Figure 6 also shows that in model A the fraction of BR which entered photocycle I decreased with pH in a way suggestive of ionization of a group with a pK higher than 10 [consistently with Hanamoto et al. (1984)].

Similarly, Figure 7 shows the pH dependencies of the rate constants in model B calculated for fits such as shown with dotted lines for three pH values in Figure 4. Again, distinct pH dependencies are obtained for the rate constants: most increased sharply at pH >9 (k_{KL}, k_{ML}, k_{LM}, k_M, k_{NM}), but those having to do with N decay decreased with pH (k_{NO} and k_{NB}). Rate constants for reversible reactions showed pH dependencies which were either in the opposite (k_{NO} and k_{ON}) or in the same direction (k_{ML} and k_{LM}, as well as k_{MN} and k_{NM}). These pH dependencies are consistent with data of the same kind but determined under a different set of conditions.[2]

Photoreactions of BR at Alkaline pH in the Presence of High Salt Concentration. Transient difference spectra were determined under conditions where background light will cause significant amounts of N to accumulate, i.e., at high pH and high salt concentration (Kouyama et al., 1988), in order to study the photoreaction of N. Panels B and C of Figure 1 show spectra of BR encased in polyacrylamide gel at pH 10.5, 100 mM $NaHCO_3$, in the presence of 3 M KCl, without and with HeNe laser background light, respectively. As in Figure 1A, spectra measured in a conventional spectrophotometer were compared with spectra in the optical multichannel analyzer (where every other readout, i.e., those without the flash excitation, was used for the averaging). Without HeNe laser background illumination, and at a low frequency of readout and flash excitation (0.25-Hz flash frequency, and 50-ms on-time for the measuring beam), we estimate that the spectrum measured (Figure 1B) contained only about 4% M (and some N, cf. below). Although some photoconversion was clearly achieved under these conditions, we have taken the results as controls for the next experiments, with HeNe laser background light. In these (Figure 1C), a much greater amount of M was accumulated in the photostationary state, as indicated by a considerable absorption increase near 410

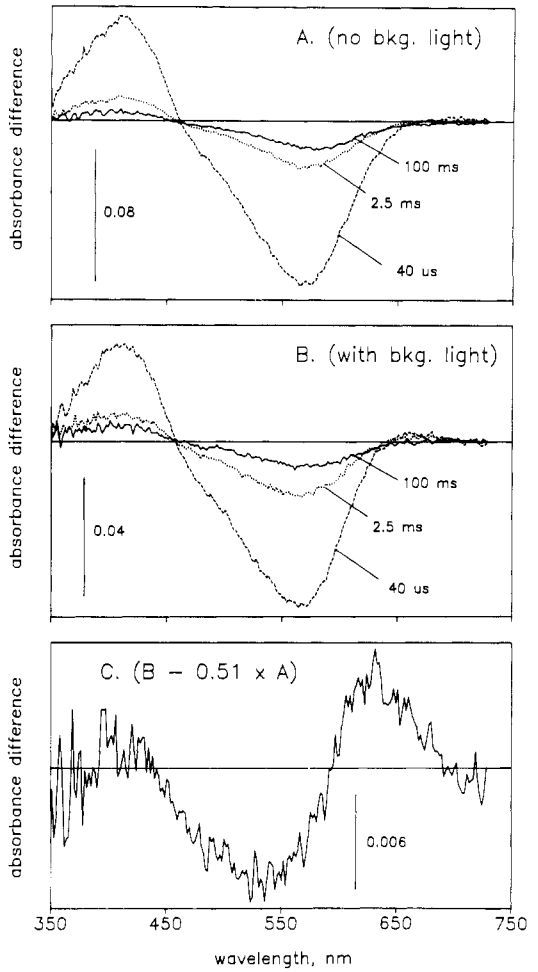

FIGURE 8: Representative difference spectra for BR at pH 10.5, 100 mM $NaHCO_3$/3 M KCl, without (A) and with (B) HeNe laser background light. Conditions as in Figure 1B,C. Delay times: (---) 40 μs; (···) 2.5 ms; (—) 100 ms. (C) Difference spectrum at 40 μs attributable to the background light only, calculated by scaling the 410-nm absorption maximum of the spectrum in (A) to that of (B) (51%), and subtracting.

nm. The amount of M was estimated as 20% by adding a scaled BR *minus* M difference spectrum until the absorption at 410 nm was restored. The resulting spectrum, also shown in Figure 1C, was then used to calculate the amount of N. By use of our earlier obtained N spectrum[2] with an extinction of 0.55 relative to that of BR, the amount of N would be 20%. According to the estimates of Kouyama et al. (1988) for the N spectrum, where the extinction is given as 0.6–0.78 relative to that of BR, higher amounts of N were calculated. It appears, therefore, that with HeNe laser illumination the photostationary state contained no more, and probably less (cf. below), than 60% remaining unconverted BR. In comparison, when the background illumination was entirely from the measuring light (Figure 1B), an estimated 90% of the BR remained in the photostationary state. We calculate that under these conditions only 6% of the BR was driven into the N state.

Panels A and B of Figure 8 show difference spectra at selected delay times after photoexcitation, without and with HeNe laser background illumination, respectively. Two differences are evident. (a) The overall amplitude of the difference spectra was diminished to about half by the background light; a decrease, although smaller, was expected from any model since the accumulated M absorbs little of the 580-nm actinic flash. (b) Net absorption increase in the red region appeared with background light, at delay times where no red-shifted intermediate could be seen otherwise (e.g., 40

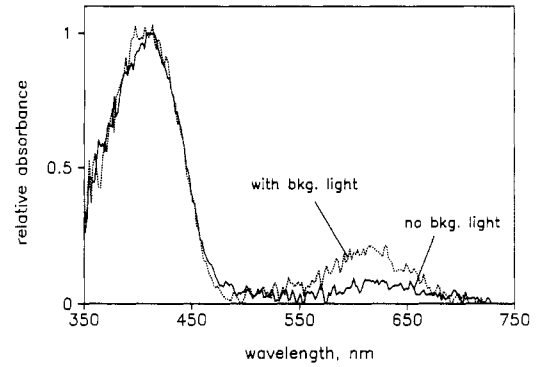

FIGURE 9: Calculated spectra of the mixtures of BR intermediates at pH 10.5, 100 mM $NaHCO_3$/3 M KCl. Delay time, 40 μs; data from Figure 8A,B. Without (—) and with (···) HeNe laser background light.

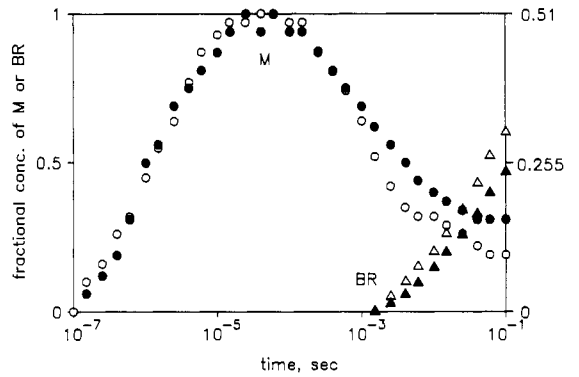

FIGURE 10: Kinetics of M and BR, shown on a logarithmic time scale, at pH 10.5, 100 mM $NaHCO_3$/3 M KCl. Points were calculated from difference spectra, such as in Figure 8. M intermediate (○, ●); BR (△, ▲); without (○, △), scale on left axis, and with (●, ▲) HeNe laser background illumination. For the latter, the scale is on the right axis.

μs). Figure 8C shows the result of subtracting a scaled 40-μs spectrum in (A) from the 40-μs spectrum in (B), so as to cancel the contribution of M near 410 nm and leave the difference spectrum of the red-shifted species *minus* its parent. Although difficult to resolve from KL in the earliest time-delay traces, this background light dependent red-shifted species seemed to appear in a constant amount in all traces obtained from 100 ns to about 1 ms (not shown). From the 2.5-ms spectrum in Figure 8B, and others not shown, we concluded that this species disappears in the 1–5-ms range.

Figure 9 shows absolute spectra for the transient mixture of intermediates at 40 μs, which contain apparently only M (normalized to the amount obtained without background illumination) and the red-shifted species. The absorption maximum of the latter is at about 610 nm. Its amount relative to the control was considerably increased by the background HeNe laser illumination: the relative amounts of the 610-nm species are roughly the same as the estimated relative contents of N in the two photostationary states (cf. Figure 1B,C), consistently with its photoproduction from N. If this is so, the quantum yield for the photoreaction of N is well below that by which M is formed from BR.

The important question is whether or not the accumulated N produces, in addition to the 610-nm species detected, also M, i.e., a slowly decaying M, as proposed by Kouyama et al. (1988). On the basis of this model, we would expect that upon flash excitation the amount of M obtained be in excess of the amount of BR in the photostationary state, the excess being the slowly decaying M produced from N. Figure 10 shows the time dependence of the rise and decay of M, and the

recovery of BR, in the absence and presence of HeNe laser background light. Intermediates KL, L, and N were also seen under both conditions, and with similar kinetics as in the absence of added salt (e.g., Figure 4), but are not shown for the sake of clarity. The amount of M present over the first 4 orders of magnitude of delay times after photoexcitation was consistently decreased to about 50% by the HeNe laser background light. Figure 10 also shows that at delay times longer than 1 ms both the decay of M and the recovery of BR were slower when HeNe laser background was applied. Replotting the data for the M decay on a linear time scale (not shown) revealed that the effect consists of an apparent increase in the amplitude of the slow decay component, as described in Figure 5 of Kouyama et al. (1988). Comparison of the amount of BR in the photostationary state and the amount of flash-produced M is difficult, because estimating the former depends on what extinction coefficient is used for N. The spectra in Figure 1C and our 0.55 relative extinction[2] for N would give 60% remaining BR, which is higher than the 51% M obtained in Figure 10. More realistically, the observed 51% M would be obtained from 51% remaining BR if the relative extinction coefficient of N were 0.63, consistent with the lower estimate by Kouyama et al. (1988). Although this seems a reasonable extinction coefficient for N, a higher value cannot be excluded, which would leave less BR than 51% in the photostationary state, and 51% *minus* this value would be the M that was produced from N. In this eventuality, the amplitude of transient M we obtained would allow the interpretation in Kouyama et al. (1988).

On the other hand, the scaled data points in Figure 10 show striking coincidence at all delay times up to 1 ms between the two experiments, deviating only at the later delay times. New rise kinetics for a putative additional M intermediate are not seen. Other than the change in decay kinetics (for which a different explanation from Kouyama's will be given below), the data in Figures 8–10 are consistent also with a simpler interpretation: that proportion of BR in the background-illuminated sample, which was not driven into the M and N states by the HeNe laser background light (i.e., 51%), produced the same reaction sequence (and with virtually unaltered kinetics, up to 1 ms) as BR without background illumination. This would account for all intermediates seen as originating from BR, but the 610-nm species. We suggest that it is the accumulated N which gives rise to this intermediate.

The change in the decay kinetics of M, reported by Kouyama et al. (1988) and confirmed here, need not arise from a second photoreaction but is explained instead by perturbation of the photostationary state. It is well-known that when there is background illumination, sufficient to drive a significant amount of a light-reactive pigment such as BR into a photostationary state, the kinetics of relaxations after additional flash excitation will be altered because they contain also the rate constant which describes the background light driven photoreaction [e.g., see Váró and Keszthelyi (1983)]. The reason for this is that the flash depletes BR, perturbing the photostationary state, and the kinetics of the relaxation to the original steady state will be mixed with the inherent kinetics of the photocycle. The slower steps of the photocycle will be more affected than the faster ones, because in the latter the inherent rates are greater. We have attempted to calculate the direction and magnitude of these effects of background illumination on the M decay kinetics, using the models in Figure 5 and the rate constants calculated from the data without background light. The calculations are described in the Appendix. Model A in Figure 5 predicted that in the photostationary state N

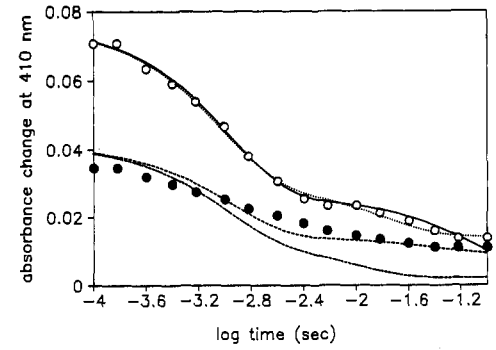

FIGURE 11: Kinetic analysis of the decay of M in Figure 10. (○) Without and (●) with HeNe laser background illumination. (—) No background illumination, model A; (···) no background illumination, model B; (– – –) background illumination, model A; (- - -) background illumination, model B.

would accumulate to 25%, while according to model B its accumulation would be 30%. Figure 11 contains, in addition to the M decay data from Figure 10 without and with background light, the lines generated by the two models. It is evident that both models accounted satisfactorily for the data without background light (as in Figure 4 under different conditions). Both models predicted that the kinetics of the M decay would be changed by the background light, but model B produced a significantly better fit. In qualitative terms, model A was unsatisfactory, because it predicted (a) the preferential depletion of BR_{II}, whose photocycle relaxes more slowly, by the background light and (b) the apparent acceleration of the M decay in photocycle I. For both of these reasons, in model A the lifetime of M decreases, a prediction in conflict with the results (Figures 10 and 11). In model B, however, the presence of N in the photostationary state contributed to the lengthening of the lifetime of M, through the N ↔ M reaction, after the flash perturbation.

It appears from these considerations, therefore, that model B accounts for the M decay kinetics both in the absence and in the presence of background illumination. In the latter case, the perturbation of the photo steady state will necessarily alter the apparent M decay, to the extent observed, without requiring photoreaction of the accumulated N. On the other hand, if model A described the BR photoreaction, the observed increase of the slow M decay component upon background illumination would require an additional explanation, such as the $N \xrightarrow{h\nu} M$ reaction proposed by Kouyama et al. (1988). However, this alternative leads to a contradiction: in model A, the amount of unconverted BR in the photostationary state is 55%, which should have produced more than the 51% M obtained, leaving a deficit, rather than an excess, of M to be produced from N. For this reason, we suggest that unless both models in Figure 5 are proved to be wrong, evoking a two-photon mechanism to explain the M decay kinetics at alkaline pH is unnecessary.

Discussion

Kouyama et al. (1988) proposed a mechanism for generating the slowed kinetics for the decay of M, observed at high pH, by postulating that a second M species is produced upon photoreaction of the intermediate N. Using conditions where significant steady-state accumulation of N (or other BR intermediates), by either thermal or photoreaction, did not occur, we found that the kinetics of M still changed as the pH was increased. Thus, it seems that the rapidly rising and slowly decaying M kinetics originate directly from BR. The kinetics of the rise and decay of intermediates L, M, and N plus O became complex as the pH was raised from 7 to 10.5 (Figure

4). The data were consistent with two kinds of photocycle schemes (Figure 5), one consisting of two parallel photocycles and another where the complex data were explained by two reversible reactions in a single photocycle. Although the molecular events which would underlie the two schemes naturally differ, and understanding of the BR photocycle requires that one of these schemes be conclusively ruled out, the pH dependencies of the individual rate constants showed consistencies not very dependent on the choice of models.

Most of the rate constants exhibited strong pH dependencies (Figures 6 and 7). Some previous reports on the pH independency of some of the kinetic steps in the BR photocycle (e.g., the decay of M) did not include data at high enough pH (Holz et al., 1989). Several reports [e.g., on the increase in the decay rate for M^f and the prominent decrease for M^s with pH (Ormos et al., 1985; Kouyama et al., 1988)] contain data similar to ours; others are to various extents in conflict (Konishi & Packer, 1978; Hanamoto et al., 1984). When the step in question is identified as the deprotonation or reprotonation of the Schiff base or a protein residue, it is tempting to attribute observed pH effects to the ionization of the proton acceptor or donor groups. This is likely to be the origin of the reciprocal pH dependency we found for the forward and reverse rate constants of the N ↔ O reaction in Figure 7. However, this is the only instance where such a result was obtained. Most rate constants in both models showed sharp increases at alkaline pH, even when they represented forward and reverse reactions (Figures 6 and 7). Such global pH dependency is likely to reflect a conformational transition. The pH at which this occurred suggests that the ionization of tyrosine(s) may play a role. Tyrosine was explicitly implicated by Braiman et al. (1988) in the BR → K and the L → M reactions as proton acceptor and donor, respectively, but these steps alone would not produce all of the pH dependencies we observe. A few of the rate constants decreased with pH in both models, all involving reactions originating from N (Figures 6 and 7).

It should be noted additionally that in model A the rate constants for the conversion of M^f and M^s into their respective N species did not show the reciprocal dependency on pH, expected from the gross pH dependencies of the two kinetic components of M decay: k_{MN}^{I} and k_{MN}^{II} both increased sharply at pH >9 (Figure 6). Thus, in this model, the observed slower decay for M^s is not the result of an inherently more stable state of M but a consequence of the increased rate of the back-reaction, N → M, at the higher pH. In model A, this happens because k_{NM}^{II} increases with pH, while k_{NB}^{II} decreases. In model B also, the slower M decay is caused by the increased rate of the net back-reaction, N → M, the result of k_{NM} increasing with pH, while k_{NB} decreases (Figure 7) and causes accumulation of N and the ensuing lengthening of the M decay.

Both models contain, as a novel feature, the reversible reaction M ↔ N which produces the observed M decay kinetics, but they differ in the way they account for the rise of M. Kinetic analysis of the data in Figure 4 could not decide between two fundamentally different models: model A with two independent reactions, $L_I \rightarrow M_I$ and $L_{II} \rightarrow M_{II}$, and model B with the sequence $L \leftrightarrow M_I \rightarrow M_{II}$. Detection of two distinguishable M forms would not necessarily decide between these alternatives either, because in both versions an initial M state is followed by a second M state. We wish to emphasize that models A and B in Figure 5 are given as representative solutions. Other schemes, which combine features of these, cannot be excluded. For example, an L ↔ M reaction (as in model B) could be followed by a branching reaction producing two M species which would decay independently (as in model A). However, to the extent that additional evidence in this report favors model B as drawn in Figure 5, we prefer it to model A and to more complex alternatives at this time. Additionally, model B has the potential to account for results in other work otherwise not easily interpreted. An example is the variability of the extent of electrical charge translocation associated with the M intermediate with externally applied electric field (Groma et al., 1988), an effect readily explained by the reversible L ↔ M reaction.

The apparent reversibility of reactions in the BR photocycle (Figure 5) affects the way the coupling of the light-induced retinal configurational transitions to the proton translocation should be viewed. As more and more of the spectroscopic transitions are found to be reversible, the irreversible "switch" event, where the energy transfer occurs, is restricted to fewer and fewer alternatives. In model B, it would have to be at the $M_I \rightarrow M_{II}$ transition, i.e., while the Schiff base is deprotonated and the retinal chain is still in the 13-cis configuration. This implies that the switch consists of either retinal motions other than isomerization around the 13–14 double bond (Oesterhelt & Tittor, 1989) or a conformational change in the protein. A protein conformational change is required by a model proposed recently (Fodor et al., 1988). Model A allows more alternatives for the localization of the switch in the reaction sequence but raises questions about whether or not both cycles translocate a proton.

Results under conditions where N will demonstrably accumulate (cf. Figures 1C and 8–10) suggest that the photoreaction of N need not contribute to M. Rather, the results are accounted for satisfactorily by the prediction based on what should happen when the flash perturbs the photostationary state produced by the background light, provided that model B in Figure 5 is used. To this extent, model B is supported by more data than model A, and we suggest, tentatively, that it describes the BR photocycle.

N appears to produce a red-shifted photoproduct with an absorption maximum near 610 nm (Figure 9), whose lifetime extends from <100 ns to a few milliseconds. Thus, the N photoreaction bears some resemblance to the photocycle of 13-*cis*-BR, in which the only long-lived intermediate detected exhibited a difference absorption peak at 610 nm (Dencher et al., 1976; Kalisky et al., 1977). At room temperature, the latter arose in <50 ns (Sperling et al., 1977) and decayed with a time constant of 37 ms (Dencher et al., 1976). The resemblance of the photoreactions of N and 13-*cis*-BR is not surprising, considering that N also contains 13-*cis*-retinal (Fodor et al., 1988), and its absorption spectrum is not far from that of dark-adapted BR (Kouyama et al., 1988; Lozier et al., 1975; footnote 2). However, this comparison should not be taken too seriously because 13-*cis*-BR contains C=N *syn*-retinal (Harbison et al., 1984), while N contains the C=N anti configuration (Fodor et al., 1988), and on this basis, their photoreaction can be expected to differ.

Another question is the effect of the photoreaction of N on proton transport. Recently (Kouyama & Nasuda-Kouyama, 1989) reported on experiments in which the effects of alkaline pH and high light intensities on proton transport in *Halobacterium halobium* envelope vesicles were described. At low pH, low light intensities, and low temperature, transport was light-limited, but when these parameters were increased, transport became limited by the slowed-down relaxation of the photocycle. Although from such a result one would normally argue in favor of single-photon-driven transport, the authors calculated that more protons were released at the most extreme

conditions than the turnover of the single-photon cycle had allowed. Hence, they suggested that the two-photon cycle also transports a proton; i.e., the light-induced N → BR transition does not inhibit the transport, as does, for example, the light-induced M → BR transition (Karvaly & Dancsházy, 1977; Dancsházy et al., 1978; Litvin et al., 1981). Our results do not contradict the idea that recovery of BR in the photocycle is accelerated by absorption of a second photon by N, and thus increase proton transport under conditions when it is photocycle-limited, since this does not depend on the photoproduction of an M from N.

ACKNOWLEDGMENTS

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APPENDIX

In the rate equations which describe model A in Figure 5, the flash-induced accumulation of the O intermediate was ignored, since at pH >7 the amount of O was negligible. With this approximation, the equations are as follows:

$$dL_I/dt = k_{KL}^{I}K_I - k_{LM}^{I}L_I \quad (A1)$$

$$dM_I/dt = k_{LM}^{I}L_I - k_{MN}^{I}M_I \quad (A2)$$

$$dN_I/dt = k_{MN}^{I}M_I - k_{NB}^{I}N_I \quad (A3)$$

$$dL_{II}/dt = k_{KL}^{II}K_{II} - k_{LM}^{II}L_{II} \quad (A4)$$

$$dM_{II}/dt = k_{LM}^{II}L_{II} + k_{NM}^{II}N_{II} - k_{MN}^{II}M_{II} \quad (A5)$$

$$dN_{II}/dt = k_{MN}^{II}M_{II} - (k_{NM}^{II} + k_{NB}^{II})N_{II} \quad (A6)$$

The rate constants are defined in Figure 5A. K, L, etc. refer to the instaneneous concentration of the intermediates. As before (Zimányi & Lanyi, 1989), the equations were solved by the numerical method, with the condition that at $t = 0$, K_I and K_{II} equal f and $1 - f$, respectively, and the concentrations of all other intermediates equal zero. Thus, f is either $[BR]_I/[BR]_{total}$ (multiplied by the quantum yields), as implied in Figure 5A, or the branching ratio in favor of photocycle I, if there is only one BR species but K is allowed to enter into either photocycle I or photocycle II. The measured amounts of L, M, and N are the sums of L_I and L_{II}, M_I and M_{II}, and N_I and N_{II}, respectively.

The equations which describe model B in Figure 5 are as follows:

$$dL/dt = k_{KL}K + k_{ML}M_I - k_{LM}L \quad (A7)$$

$$dM_I/dt = k_{LM}L - (k_{ML} + k_M)M_I \quad (A8)$$

$$dM_{II}/dt = k_M M_I + k_{NM}N - k_{MN}M_{II} \quad (A9)$$

$$dN/dt = k_{MN}M_{II} + k_{ON}O - (k_{NM} + k_{NO})N \quad (A10)$$

$$dO/dt = k_{NO}N - (k_{ON} + k_{OB})O \quad (A11)$$

The rate constants are defined in Figure 5B. The initial condition for this model was that at $t = 0$, $K = 1$, and the concentrations of all other intermediates equal zero.

For the calculations with background light at pH 10.5 (Figures 8–11), only the decay of M was considered. The rate equations were used both for following the establishment of the photostationary states and for the relaxations that followed the flash excitation. For the former, the initial condition was that at $t = 0$, M and N are zero, and [BR] = 1. For the latter, the initial condition was that at $t = 0$, M (or M_I *plus* M_{II}) is the photostationary amount of M *plus* the additional photoexcitation, N equals the photostationary concentration, and [BR] is the photostationary amount of BR *minus* the amount excited by the flash. For model A, eq A3 and A6, as well as eq A12 and A13, were used where $k_{h\nu}f$ and $k_{h\nu}(1 - f)$ are the

$$dM_I/dt = k_{h\nu}f[BR] - k_{MN}^{I}M_I \quad (A12)$$

$$dM_{II}/dt = k_{h\nu}(1 - f)[BR] + k_{NM}^{II}N_{II} - k_{MN}^{II}M_{II} \quad (A13)$$

rates at which M_I and M_{II} are generated, respectively, by the background illumination. As mentioned previously, any differences in the quantum yields of the two photocycles are included in f and $1 - f$. As before, $M_I + M_{II}$ equals the measured amount of M, and likewise for N.

For model B under these conditions, eq A10 and A11, as well as eq A14, were used where $k_{h\nu}$ is the rate at which M_{II} is generated by the background illumination.

$$dM_{II}/dt = k_{h\nu}[BR] + k_{NM}N - k_{MN}M_{II} \quad (A14)$$

For both models, first the results without HeNe laser background illumination were fitted, after several iterations, so that (a) the photostationary state contained 4% M (Figure 1B) and (b) the decay of M after the flash was satisfactorily described (Figure 11). From both models, $k_{h\nu} = 2\ s^{-1}$ was obtained, and the rate constants (and an f value) were similar to those in Figures 6 and 7 at high pH. Then, by use of the two sets of rate constants in the two models, the $k_{h\nu}$ values for the background light experiments were adjusted until the photostationary amount of M reached 20% (Figure 1C). From model A, $k_{h\nu} = 50\ s^{-1}$, and for model B, $k_{h\nu} = 10\ s^{-1}$ was obtained. With these values, the photostationary amounts of N could be calculated. Once these were known, a prediction for the M decay, shown in Figure 11, was generated from each of the models.

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