Protonation and Deprotonation of the M, N, and O Intermediates during the Bacteriorhodopsin Photocycle†

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ABSTRACT: Transient pH changes were measured with phenol red and chlorophenol red in the 30-μs–50-ms time range during the photocycle of bacteriorhodopsin (BR), the light-driven proton pump. At pH ≥7, the results confirmed earlier data and suggestions that one proton is released during the L → M reaction, and taken up again during the decay of N. These are likely to be steps in the proton transport process. At pH <7, however, the time-resolved pH traces were complex and indicated additional protonation reactions. The data were explained by a model which assumed pH-dependent protonation states for M and N which varied from −1 to 0, and for O which varied from 0 to +2, relative to BR. If the kinetics of the vectorial proton translocation process were taken as pH independent, this treatment of the data suggested that a residue with a pK_a of 5.9 was made protonable in M and N and two residues with pK_a's of 6.5 were made cooperatively protonable in O. The additional protons detected are not necessarily in the vectorial proton transfer pathway (i.e., they are probably "Bohr protons"), and while they must reflect conformational and/or neighboring ionization changes in the BR as it passes through the M, N, and O states, their role, if any, in the transport is uncertain.

In bacteriorhodopsin (BR),[1] the light-driven proton pump of the cytoplasmic membrane of halobacteria, the translocation of protons across the membrane, is coupled to the transient all-trans to 13-cis isomerization of the retinal. The reaction cycle ("photocycle"), initiated by absorption of a photon, and completed in about 10 ms near neutral pH, is described by the sequence of the rise and decay of the spectroscopically distinct species designated as J, K, KL, L, M, N, and O [for reviews, see Stoeckenius et al. (1978), Stoeckenius and Bogomolni (1982), and Lanyi (1984)]. Of these species, M assumes special significance because it contains a deprotonated retinal–lysine Schiff base. The loss of the Schiff base proton upon formation of M, and its regain during its decay, is thought to proceed via different routes defined by separate proton acceptor and donor residues of the protein, implicating these reactions directly in the "switch" which determines the direction of the proton translocation.

It has long been known that during the BR photocycle first proton release and later proton uptake will be observed. In membrane vesicles, the released protons appeared on the extracellular side of the membrane, and those taken up disappeared from the cytoplasmic side (Lozier et al., 1976), as required by net proton transport in this system. The first question concerning these protons was their stoichiometry, i.e., the number of protons released for every cycling BR. One way to calculate this was from the amount of M produced (estimated from the transient absorption change at 410 nm) and the amount of protons released (estimated from the transient absorption change of a pH indicator, or in one case from volume changes). While recognizing that in a single-compartment system, such as the usually employed BR-containing purple membrane sheets, protons released and taken up on the same side ("Bohr protons") cannot be distinguished from protons translocated across the membrane, the number of transiently released protons was reported to be between 1 and 3, depending on the salt concentration (Lozier et al., 1975, 1976; Ort & Parson, 1978; Govindjee et al., 1980; Kuschmitz & Hess, 1981). It is now known that stoichiometries larger than 1 were due to calibration errors arising from the slowness of buffering by the CO_2/H_2CO_3 equilibrium in purple membrane suspensions which were not degassed (Drachev et al., 1984). The second issue was the correlation of the kinetics of proton release and uptake with the kinetics of the BR intermediates. It was reasonable, but not absolutely necessary, to expect that the protonation changes of BR would occur at the same times as transformations detected by spectroscopic changes in the chromophore. In fact, the proton release was seen to either coincide with (Drachev et al., 1984) or lag somewhat behind (Grzesiek & Dencher, 1986) the rise of M. A likely source of error was that the rate of proton transfer from the surface of BR to the dye was a limiting factor, since the measured rates were accelerated in the presence of buffer (Drachev et al., 1984; Grzesiek & Dencher, 1986). For this reason, it is now generally held that proton release into the medium is concurrent with the formation of M; i.e., it is initiated when the proton leaves the Schiff base. Although a buffer effect was not seen for the rate of proton reuptake, it followed not the time course of the entire M decay but its slower phase (Grzesiek & Dencher, 1986). Photoelectric measurements had also suggested that the last stage of charge (proton) transfer occurs after the M decay [reviewed recently in Keszthelyi and Ormos (1989)]. Since the M → (N, O) → BR reaction sequence was poorly defined, little could be said about the exact relationship of proton uptake kinetics of these intermediates. However, recently Skulachev et al. (1988), Kouyama et al. (1988), Kouyama and Nasuda-Kouyama

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[1] Abbreviations: BR, bacteriorhodospin; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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(1989), and Otto et al. (1989) proposed that reprotonation is during the decay of N (which the former authors called P).

The residues involved in these proton transfer reactions have been, for the most part, identified. Early FTIR results (Engelhard et al., 1985; Eisenstein et al., 1987; Engelhard & Hess, 1987) suggested changes in the protonation state of specific aspartate residues during the photocycle. More recent FTIR results with BR mutants (Braiman et al., 1988; Gerwert et al., 1989) and studies of chromophore transformations in the visible and of transport (Butt et al., 1989; Stern et al., 1989) identified Asp-85 as the acceptor of the Schiff base proton during the L → M reaction and implicated perhaps also Arg-82 in proton release. The residue that reprotonates the Schiff base is established as Asp-96. Time-resolved FTIR spectra showed that Asp-96 deprotonated together with the decay of M (Gerwert et al., 1989). Its replacement with nonprotonable residues slowed down the decay rate of M and introduced a considerable pH dependency to it (Butt et al., 1989; Holz et al., 1989; Tittor et al., 1989; Otto et al., 1989; Stern et al., 1989), suggesting that M must be now reprotonated from the medium. Tittor et al. (1989) found that a weak acid like azide greatly accelerated the M decay in the mutant, acting apparently as an artificial proton donor, and restored transport activity. This experiment was repeated by Otto et al. (1989). Thus, much evidence suggests that in BR the proton uptake near the end of the photocycle is the reprotonation of Asp-96. Otto et al. (1989) proposed a more explicit model: the M → N reaction consists of the transfer of a proton from Asp-96 to the Schiff base, and the N → O reaction consists of the reprotonation of Asp-96. Although its direct role in the vectorial proton transfer chain is uncertain, FTIR spectra suggested also the protonation of Asp-212 during the formation of M (Braiman et al., 1988; Gerwert et al., 1989). Likewise, any direct role for tyrosine(s) in the transport is uncertain, but deprotonation of a tyrosine during the formation of M was suggested on the basis of UV difference spectra (Bogomolni et al., 1978; Hess & Kuschmitz, 1979; Hanamoto et al., 1984).

In fact, the protonation state of BR changes in a more complex way during the photocycle than implied by the model containing only proton release from Asp-85 followed by proton uptake by Asp-96. It has been known for some time (Garty et al., 1977; Fischer & Oesterhelt, 1980; Takeuchi et al., 1981; Renard & Delmelle, 1985) that whether protons are released or taken up by the BR intermediates which accumulate during sustained illumination depends on the pH: above about pH 6, there is net deprotonation, but below there is net proton uptake. Indeed, time-resolved pH measurements at lower pH with dyes (Dencher & Wilms, 1975; Marinetti & Mauzerall, 1983; Sinton & Dewey, 1988) and a fast pH electrode (Bogomolni et al., 1986) detected this uptake of proton(s). At the same time, under carefully defined conditions, BR is known to transport protons at a virtually constant rate between pH 4 and 9 (Kouyama & Nasuda-Kouyama, 1989; Vârô et al., 1990). It seemed to us that correlating the time-resolved protonation kinetics of BR at varying pH with the appearance and disappearance of intermediates would be informative, since we have recently proposed a model for the reaction pathway from M to BR via N and Q in the pH region 4–7 (Vârô et al., 1990), and a general model for the M relaxation up to very high pH has been proposed independently by us [model B in Vârô and Lanyi (1990)] and by Otto et al. (1989).

Materials and Methods

Purple membrane was prepared from *Halobacterium halobium* strain S9, as previously described (Oesterhelt & Stoeckenius, 1974). All samples prepared for the measurements contained 100 mM KCl. The bacteriorhodopsin concentration was 25–35 nmol/mL; buffer was added only when so stated. All solutions used were thoroughly degassed and kept in a nitrogen atmosphere over NaOH pellets, in order to avoid absorption of CO_2 from the air. For the same reason, Teflon-stoppered cuvettes, nearly filled to the top and closed under nitrogen, were used. The pH was monitored in these samples throughout the experiments by repeatedly measuring dye absorbance, and results were accepted only when the pH did not change appreciably between the first measurement and the last. All experiments were at 22 °C, and after light adaptation of the BR samples. The latter was checked by measuring BR absorbance repeatedly throughout the experiments. The optical path length used was 4 mm, unless stated otherwise. Stationary spectra and optical densities were determined with a Shimadzu UV-250 split-beam, double-monochromator spectrophotometer, connected to an XT desk-top computer.

Measurements of BR intermediate and dye kinetics were with a split-beam transient spectrometer described earlier (Duschl et al., 1988). The flash was provided by a Photochemical Research Associates (London, Ontario, Canada) LN 1000/LN102 nitrogen laser/dye laser combination (tuned to 610 nm). Averaging (128 or 256 sweeps) was into a Data Precision Corp. (Danvers, MA) Model DATA 6000 signal acquisition recorder, over 2 overlapping time ranges (512 points at dwell times of 10 and 100 μs). The time constant of the measuring system was 30 μs. Timing of the flashes (about 1 Hz) was from the laser; signal acquisition was initiated from an optical trigger activated by the flash. A base line was provided by setting a negative delay of 0.4 or 4 ms. The laser intensity was monitored with a Gentec Inc. (Palo Alto, CA) Model ED-100A pyroelectric joulemeter, whose amplified output during the measurements was averaged into a second, parallel channel of the DATA 6000. The transient measurements were followed by calibration, which consisted of three 1-μL additions of 2.5 or 10 mM HCl and optical density determinations at 570 nm. The completed files were transferred into an AT-286 desk-top computer and imported into spreadsheets for data reduction, in which, when required, the time scale was converted from linear to logarithmic.

The pH changes during sustained illumination of purple membrane suspensions were recorded with a glass electrode, in a thermostatted (22 °C), stirred cuvette and illumination system (actinic light > 530 nm) described before (Duschl et al. 1988). As above, calibration was with HCl additions.

Results

pH Changes in Photostationary States. Earlier work (Garty et al., 1977; Fischer & Oesterhelt, 1980; Takeuchi et al., 1981) had indicated that during sustained illumination BR intermediates accumulated in sufficient quantities to measure a protonation change with a glass electrode: at higher pH, there was net proton release from BR, as expected from the time-resolved pH measurements, but at lower pH, there was net proton uptake. We have repeated these experiments in the pH range of 3–8, under the conditions used in the rest of this work, and confirmed the results. Figure 1 shows that in the presence of 100 mM KCl there was a pH at which no net light-induced protonation change of BR occurred upon illumination, i.e., pH 5.0. Above this, there was a continuous increase in light-induced proton *release* with increasing pH, and below, there was continuous increase in proton *uptake* with decreasing pH. In these measurements, there was no information as to the kind and amount of intermediates which

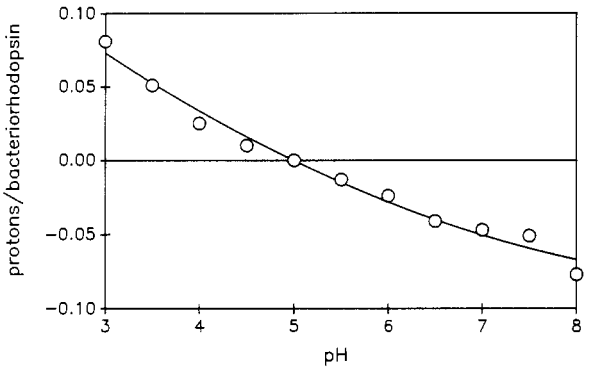

FIGURE 1: Proton release and uptake from BR during photostationary states at different pHs. The conditions for measurement of pH changes with a glass electrode during the sustained illumination of a purple membrane suspension are described under Materials and Methods. Positive numbers indicate proton uptake, negative numbers proton release, divided by the total amount of BR present. Measurement error: ±0.01 H^+/BR.

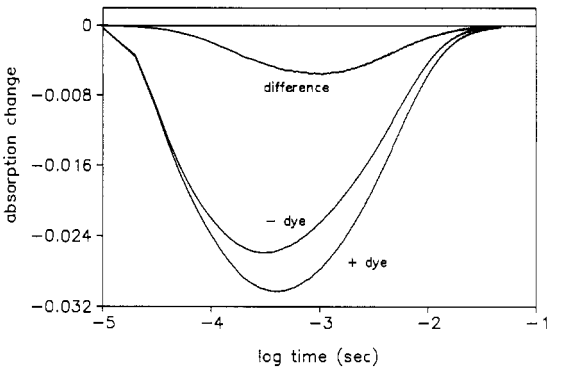

FIGURE 2: Flash-induced absorption change of a BR sample with and without phenol red, and the difference. The transient absorption changes were measured at 570 nm, as described under Materials and Methods.

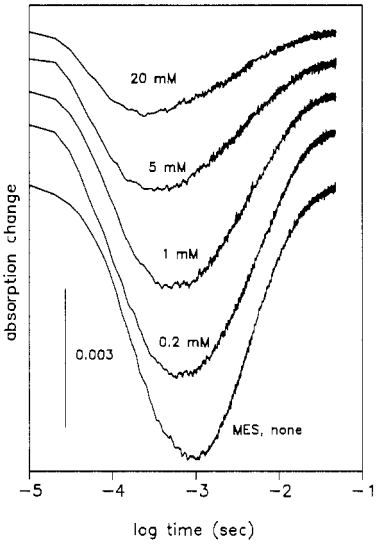

FIGURE 3: Flash-induced transient absorption changes in BR samples at 570 nm, due to pH changes detected by phenol red. The measurements were at pH 7.0, with increasing amounts of buffer (MES) present as indicated.

accumulated (mostly M but also N and O, in amounts dependent on the pH and light intensity). Thus, the positive and negative protonation changes, which reached maximal values of about 0.075 H^+/BR under the illumination conditions used (Figure 1), could not be related to intermediates of the photocycle. The qualitative conclusion could be drawn, however, that measurements of time-resolved pH changes solely at pH 7 would reveal only part of a complex process.

Description of Phenol Red and Chlorophenol Red as Indicator Dyes. As stated earlier (Lozier, 1982), an ideal dye for time-resolved pH determinations will (1) have a pK_a not far from the pH of the measurement, (2) show maximal absorption change at a wavelength near an isosbestic point of the BR photocycle, (3) not absorb much at the wavelength of the exciting light pulse, and (4) not bind to BR. Dyes used before, i.e., *p*-nitrophenol (Lozier et al., 1976; Govindjee et al., 1980; Drachev et al., 1984), brilliant yellow (Lozier et al., 1975), 7-hydroxycoumarin (Lozier et al., 1976), and pyranine (Grzesiek & Dencher, 1986), did not satisfy criterion 1 for us, inasmuch as we wished to cover a large pH range. The dye pair we used, phenol red (phenolsulfonephthalein) and chlorophenol red (3′,3″-dichlorophenolsulfonephthalein), did not satisfy criterion 2 since their absorbance maxima were at 559 and 575 nm, respectively, but we accepted this drawback in view of advantages on the other points.

The measured pK_a's of chlorophenol red and phenol red under our experimental conditions were 6.0 and 7.8, respectively (not shown). We used these dyes at or below their pK_a's in all experiments, except in one case (at pH 8.5), and added them at each pH in amounts to give absorbances of 0.3 at the maximum (4-mm path length). This ensured that the absorbance at the exciting wavelength of 610 nm was only 0.07 for chlorophenol (<2% screening of the flash within the 1-mm pathway between the cuvette wall and the measuring beam) and less for phenol red, and that the absorbance changes for a given pH change were similar at each measurement pH.

The dye do not bind significantly to BR. This was shown by an experiment where BR and phenol red were mixed in equimolar concentrations (30 μM each) at pH 7.5, with 20 mM MES and 100 mM KCl present. Calculations from absorption spectra indicated that the supernatant after removal of BR by centrifugation contained essentially all (within ± 2% error) of the added dye.

Time-Resolved pH Measurements. Figure 2 shows the absorption change of a BR sample at 570 nm, at pH 7.0, with and without phenol red, and the difference. Displaying the time after the flash on a logarithmic scale allowed evaluation of the rise phases of the changes, as well as the decays. It is evident from Figure 2 that in spite of the large absorption change of BR, the change in dye absorption, with kinetics differing from those of BR at this wavelength, was well resolved.

The effect of buffer (MES) on the magnitude and kinetics of dye absorption changes at pH 7.0 is shown in Figure 3. Increasing amounts of buffer decreased the absorption change, as expected, and accelerated the proton release kinetics without significantly altering the uptake kinetics. Thus, with more than 5 mM MES, the estimated time constant of the release decreased from 220 μs to 80–100 μs, while that of the uptake changed from 8 to 6 ms. Similar results were seen at lower pH with chlorophenol red as indicator (not shown). These results on the influence of buffer on the kinetics thus confirmed earlier reports (Drachev et al., 1984; Grzesiek & Dencher, 1986), where the apparently more effective proton conduction from BR to the bulk phase by buffers accelerated the submillisecond protonation of the dye used, but not its deprotonation at longer times. While our estimated uptake time constant agrees reasonably with earlier results with pyranine under similar conditions [11.3 ms in Grzesiek and Dencher (1986), with or without buffer], our release time constant is faster [646 μs in Grzesiek and Dencher (1986), without buffer, and 332 μs with buffer]. The reason may be that phenol red

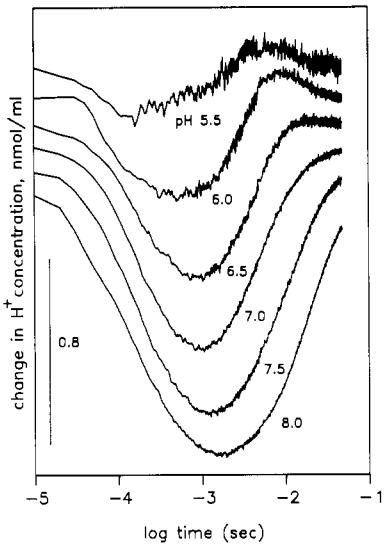

FIGURE 4: Flash-induced transient changes in proton concentration in BR samples, detected by chlorophenol red or phenol red, at various pHs, as indicated. Downward deflection indicates proton release from BR.

contains fewer negative charges, and is not excluded from the BR surface as much as pyranine. This may explain also why the signals in Figure 3 were not as sensitive to buffer as for pyranine. With phenol red, the measured rate of proton release in the presence of buffer approached, within experimental error, the rate of M formation (about 100 μs, cf. below). From the absorption changes of the dye (without added buffer) and the amounts of BR which had entered the photocycle (cf. below), the proton/photocycle ratio was calculated to be 0.8 ± 0.1. This agrees with the 0.7 ± 0.1 H^+/BR reported by Drachev et al. (1984). It should be noted that scaling the traces in Figure 3 together, so as to make the decays coincide (not shown), indicated that the slow rate at which the release of the protons was detected had decreased the maximal amplitude that would be seen for the released protons to about 75%. Thus, a detected stoichiometry of 0.7–0.8 corresponds to an actual stoichiometry of about 1 H^+/BR.

The measurement of transient pH change was then repeated at various pH values, between 5.5 and 8, using chlorophenol red up to pH 6.0, and phenol red above. The time courses of these difference traces, expressed as protons (nanomoles per milliliter) removed from BR, are shown in Figure 4. It is evident that, as expected from the steady-state results in Figure 1, the quantity of transient protons detected decreased steeply with decreasing pH. Furthermore, the shape of the traces became complex. As the pH was lowered, the net release of protons occurred at progressively earlier times, shifting from about 1.7 ms at pH 8 to about 200 μs at pH 5.5. In addition, as the pH was lowered, proton uptake was increasingly seen after the transient release, resulting in *net* uptake near 5–10 ms at pH 6 and below. At pH 5 and below, the traces were even more complex, and in addition to progressively increasing net proton uptake which replaced net proton release, a second dye absorbance decrease appeared near 50 ms (not shown). The latter was not removed by buffering like the changes at higher pH, and its origin is uncertain. It was not explored further in this work. Interpretation of the complex transient protonation changes in Figure 4 required that we describe the kinetics of the photointermediates under the conditions of these pH measurements.

Time-Resolved Measurement of the BR Intermediates. The transient concentrations of the intermediates in the second half of the photocycle could be calculated from time-dependent

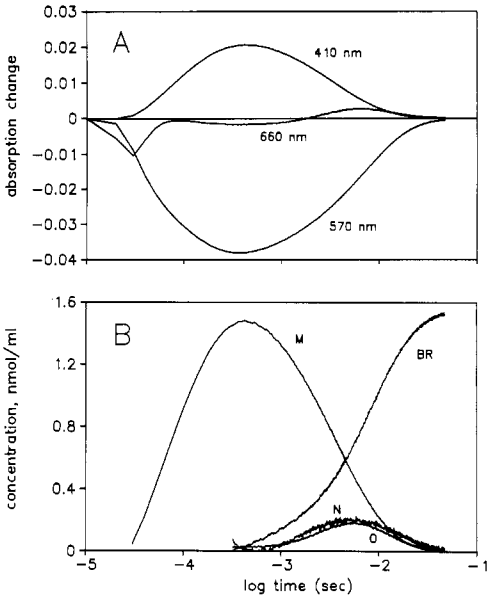

FIGURE 5: Kinetics of the BR photocycle at pH 7.0. (A) Absorption changes at 410, 570, and 660 nm. (B) Concentrations of M, N, and O, calculated with eq 1–4. The curve labeled as BR refers to the total amount of pigment which had entered the photocycle *minus* the concentration which had not yet returned to BR at time t.

absorption changes at three wavelengths (660, 570, and 410 nm). The following equations were used:

$$\Delta A_{660}(t) = \epsilon^{O}_{660} O(t) - \epsilon^{BR}_{660}[BR](t) \quad (1)$$

$$\Delta A_{570}(t) = \epsilon^{O}_{570} O(t) + \epsilon^{N}_{570} N(t) - \epsilon^{BR}_{570}[BR](t) \quad (2)$$

$$\Delta A_{410}(t) = \epsilon^{N}_{410} N(t) + \epsilon^{M}_{410} M(t) - \epsilon^{BR}_{410}[BR](t) \quad (3)$$

$$[BR](t) = O(t) + N(t) + M(t) \quad (4)$$

where the ΔA's are the time-dependent absorption changes at the wavelengths indicated with subscripts, the ϵ's are the extinction coefficients of the superscripted species at the wavelengths of the subscripts, $[BR](t)$ is the time-dependent concentration of BR in the photocycle, and $O(t)$, $N(t)$, and $M(t)$ are the time-dependent concentrations of O, N, and M, respectively. The equations assume that N and M have no significant absorption at 660 nm; likewise that M has no absorption at 570 nm, and O has no absorption at 410 nm. The solutions to eq 1–4 gave $[BR](t)$, $N(t)$, $O(t)$, and $M(t)$ in terms of absorption changes and extinction coefficients (not shown). The absorption changes were measured (cf. below), and the latter were estimated from spectra calculated from data obtained earlier with a gated optical multichannel analyzer (Vårõ et al., 1990). They were as follows: ϵ^{O}_{660} = 53 700 $M^{-1}\cdot cm^{-1}$; ϵ^{BR}_{660} = 3600 $M^{-1}\cdot cm^{-1}$; ϵ^{O}_{570} = 12 900 $M^{-1}\cdot cm^{-1}$; ϵ^{N}_{570} = 32 500 $M^{-1}\cdot cm^{-1}$; ϵ^{BR}_{570} = 63 000 $M^{-1}\cdot cm^{-1}$; ϵ^{N}_{410} = 4000 $M^{-1}\cdot cm^{-1}$; ϵ^{M}_{410} = 40 800 $M^{-1}\cdot cm^{-1}$; ϵ^{BR}_{410} = 5000 $M^{-1}\cdot cm^{-1}$. Since the absorption change at 410 nm was dependent mostly on the concentration of M, the data generated reasonably accurate $M(t)$ values for times as early as 30 μs.

Absorption changes at 410, 570, and 660 nm, and calculated transient concentrations of M, N, O, and Br at pH 7.0, are shown in Figure 5. The 660-nm absorption trace contains a small laser artifact, which appears with the time constant of the measurement. Because the spectrum of the L intermediate is similar to that of N (Kouyama et al., 1988; Vårõ et al., 1990), the calculated trace for N before maximal M had accumulated represents, in fact, the decay of L. This part of the N trace, as well as the beginning of the M and O traces, was omitted from the graphs, however (Figure 5B). The overall time constant of the rise of M was about 100 μs.

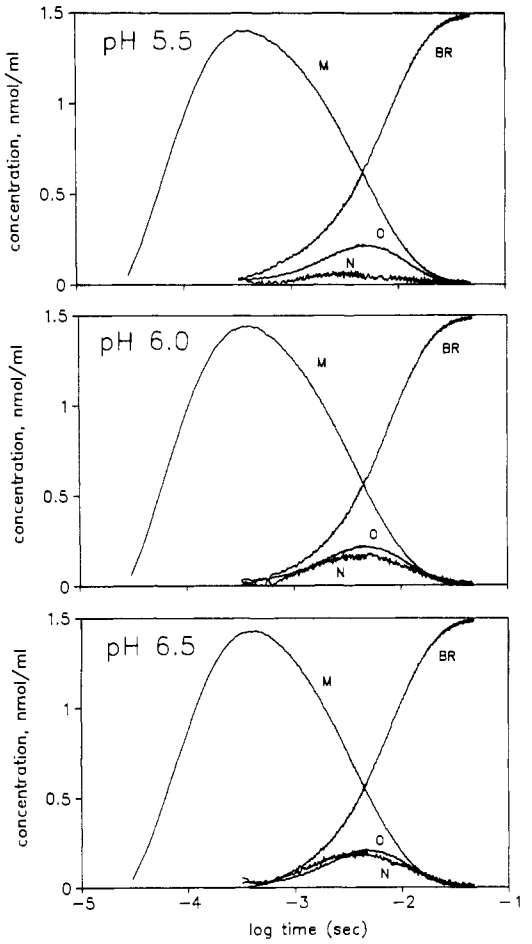

FIGURE 6: Kinetics of the BR photocycle at pH 5.5, 6.0, and 6.5. The curves for M, N, O, and BR were calculated from data at these pH values, as in Figure 5B.

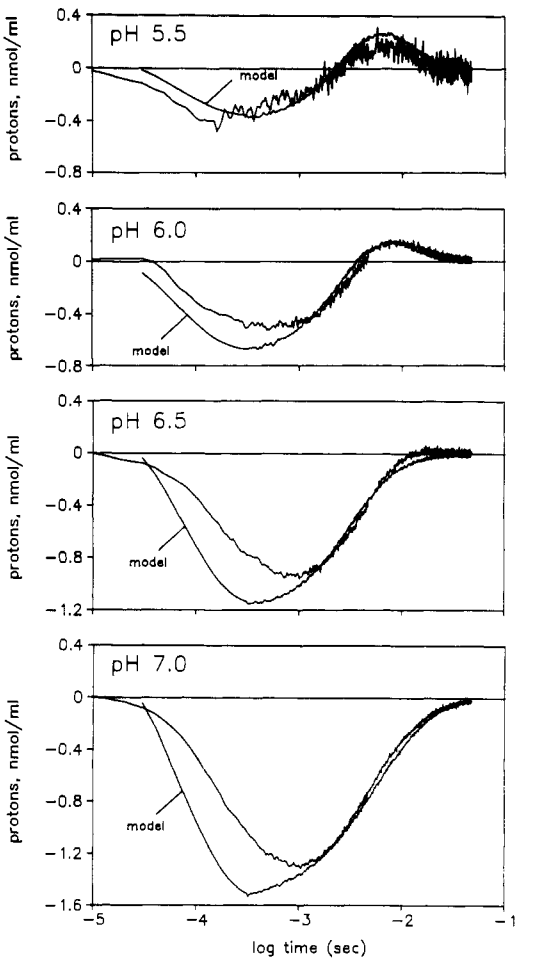

FIGURE 7: Flash-induced transient changes in proton concentration in BR samples at pH 5.5, 6.0, 6.5, and 7.0, and fitting of the curves with a model. The data are from Figure 4; the model is described in the text. The negative numbers refer to detected protons released from BR, and the positive numbers to protons taken up.

The photocycle kinetics at pH 6.5, 6.0, and 5.5, obtained in the same way as at pH 7.0, as shown in Figure 6. These traces resemble what we had seen earlier (Vārō et al., 1990) in the presence of 1 M Na_2SO_4 with a gated optical multichannel analyzer: with decreasing pH, the maximum of N accumulation decreased and shifted to earlier times. The amplitude of O, on the other hand, began to decline at pH 7 (Figure 5B), and was very small at pH 8.5 (cf. below). As with the previous data (Vārō et al., 1990), we fitted the traces to several models, and here also only a branched scheme, containing two pathways from N to BR ($O \leftrightarrow N \rightarrow BR$ and $N \leftrightarrow O \rightarrow BR$), gave satisfactory fit (not shown). By and large, the rate constants and their pH dependencies were similar to those at the high salt concentration. They indicated that, as in the previous study, at lower pH the $N \rightarrow BR$ pathway and at higher pH the linear $N \leftrightarrow O \rightarrow BR$ pathway dominated.

Fitting of Transient pH Changes to the Kinetics of BR Intermediates. As a working hypothesis, we assumed that the complex changes in the protonation of BR, shown in Figure 4, reflected solely the presence of the spectroscopically detectable, intermediates, M, N, and O (Figures 5B and 6). Thus, as M, N, and O were produced and decayed, protons were lost or gained, according to the concentrations and the protonation states of these species relative to that of BR. In this case, the protons detected by the dye would be given by $p_M M(t) + p_N N(t) + p_O O(t)$, where $M(t)$, $N(t)$, and $O(t)$ are the time-dependent concentrations of M, N, and O during the photocycle, and the subscripted p's are the protonation states of these intermediates relative to BR. For the latter, we considered the following. Results at higher pH established (cf. below) that M and N both represent deprotonated states relative to BR; this feature was retained in the model. The temporal coincidence of the net proton uptake at pH 5.5–6.0 (Figure 4) and the appearance of O (Figure 6) suggested that O is an overprotonated state relative to BR. The coincidence of the proton uptake with the appearance of O at pH 5 was noted already by Dencher and Wilms (1975). Interestingly, Takeuchi et al. (1981) could suggest also, on the basis of evidence which did not include time-resolved pH measurements, that the net proton uptake should occur at a step after M. With these ground rules, we attempted to reconstruct the shapes of the protonation curves, chosing p values which gave fits. The traces in Figure 7 show that this was possible. Here we compare the observed changes in proton concentration, and the result of the calculation which gave the best fit ("model"). For clarity, in the time domain preceding the maximum of M, we joined to the latter a trace containing only M, since here $N(t)$ and $O(t)$ were necessarily zero, even if the calculation of the $N(t)$ trace could not show that. The only significant deviations between the "model" and the experimental traces are in the submillisecond time domain, where proton conduction will limit the detection of pH changes (cf. above and references cited), and therefore the measured traces cannot be correct.

Since the protonation numbers of M, N, and O were estimated by comparisons of the model with the pH curves at times where each of these intermediates reached their own

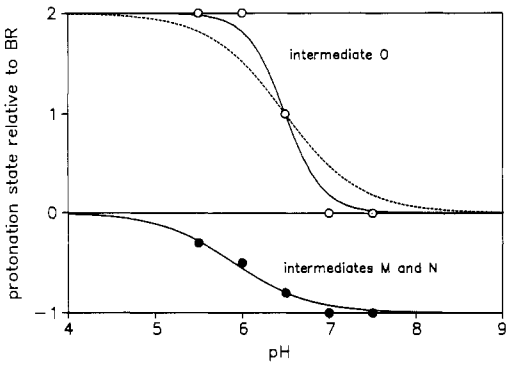

FIGURE 8: Protonation states of M and N, and O, used in the "model" curves in Figure 7. The line for M and N is the predicted protonation of a weak acid with a pK_a of 5.9. The solid line for O is the predicted cooperative protonation of two weakly acidic groups with pK_a's of 6.5. The dashed line is the predicted independent protonation of these two groups.

maximum concentrations, the numbers chosen are probably unique solutions. Keeping the protonation states constant while the pH varied did not give an acceptable solution; the values are therefore given as functions of pH in Figure 8. The fitting in Figure 7 required that the protonation state of M and N change (together) from −1 to near zero, relative to BR, along a curve which describes the protonation of a single residue with a pK_a of 5.9 (points and curve in Figure 8, lower panel). We interpret this as a pH-independent loss of one proton *plus* the pH-dependent protonation of a second residue, whose pK_a is raised into the measured range when BR passes through the M and N states. Similarly, the fitting in Figure 7 required that the protonation state of O change with pH, but from 0 to +2, and along a curve which describes the cooperative protonation of two residues, each with a pK_a of 6.5 (points and solid curve in Figure 8, upper panel). For comparison, a curve describing the independent protonation of two such residues is also included in Figure 8 (upper panel, dashed curve).

Correlation of Reprotonation Kinetics with the Kinetics of M and N at pH 8.5. Skulachev et al. (1988), Kouyama et al. (1988), Kouyama and Nasuda-Kouyama, (1989), and Otto et al. (1989) suggested that the decay of N consists of the reprotonation of BR; i.e., both M and N are deprotonated species. This possibility, and the alternative, that only M is a deprotonated state, could be most critically examined at high pH, where the M and N kinetics are considerably separated. At pH 8.5, determination of the dye kinetics had to be in the presence of added buffer, because buffering by BR was so low that the pH would not remain constant otherwise, in spite of all precautions to exclude CO_2 during the measurements. Figure 9A shows the kinetics of M, N, and O on a linear time scale, in order to emphasize the shapes of the decay curves. The second, slower component of the M decay, which proceeds with the time constant of the N decay because of equilibration of M and N (Váró & Lanyi, 1990; Otto et al., 1989), is evident at this pH. If reprotonation were during the M → N reaction, the deprotonation curve would be described by the concentration of M (multiplied by its protonation state), but if it were to occur during the decay of N, it would be described by M *plus* N (multiplied by their protonation states). We found that the deprotonation of BR was clearly coincident with the negative of M *plus* N (Figure 9B), arguing for the former alternative and for the fact that, at this pH at least, M and N have protonation numbers of −1, relative to BR.

Discussion

We have compared data on the rise and decay of intermediates M, N, and O, and the protonation state of BR in the

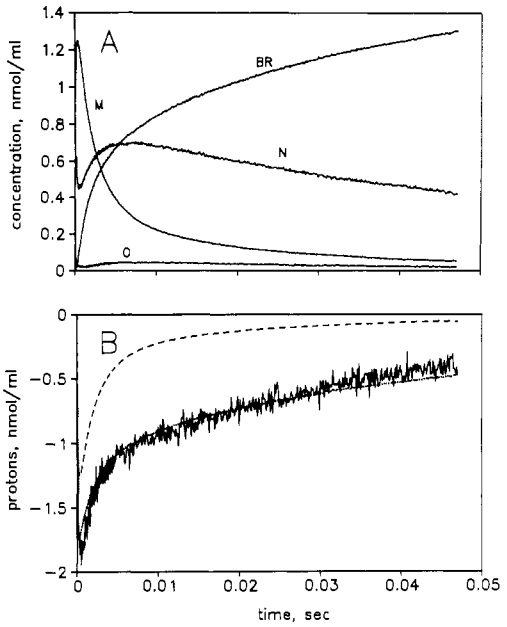

FIGURE 9: Photocycle kinetics (A) and predicted and observed proton concentration changes (B) in a BR sample at pH 8.5. In (A), the transient concentrations of M, N, O, and BR have been calculated as in Figure 5B. In (B), the solid (noisy) line is the experimental trace, determined at this pH as in Figures 4 and 7, but in the presence of 0.2 mM Tris·HCl, the dashed line is the predicted trace if M is the only deprotonated species, and the dotted line is the predicted trace if M and N are both deprotonated species relative to BR. Protonation numbers of −1 were assumed in each case.

30-μs–50-ms time range, between pH 5.5 and 8.5. This kind of data serves as the basis for formulating and testing specific models of proton transport, which relate changes in the chromophore to proton transfer events elsewhere in the protein. Importantly, we found that all proton exchange with the medium could be correlated directly with measured reactions of the chromophore. Earlier attempts of interpreting time-resolved proton kinetics were less conclusive, or required more complex models containing, for example, two parallel M intermediates (Sinton & Dewey, 1988).

The results and their interpretation were relatively simple at pH >7. After fitting various kinetic schemes to spectroscopic data obtained with a gated multichannel analyzer, we recently proposed a model for the steps leading from the M intermediate to BR, which accounted for the kinetic anomalies of the M decay at pH >7 with a reversible M ↔ N reaction [model B in Váró and Lanyi (1990)]. We found that the rate constants for the N → O and N → BR transitions were strongly dependent on the concentration of protons, while those of the M → N and N → M transitions were much less so, but did not offer a molecular mechanism to account for this. At the same time and independently, Otto et al. (1989) proposed essentially the same model from data on the relaxation kinetics of M. Importantly, however, in their model Otto et al. (1989) attributed the pH dependency of N decay (Kouyama & Nasuda-Kouyama, 1989) to the reprotonation of Asp-96 from the medium. Thus, similarly to Skulachev et al. (1988) and Kouyama et al. (1988), they proposed a two-state protonation process: in their version an intramolecular transfer of a proton from Asp-96 to the Schiff base (M ↔ N, independent of the proton concentration) was followed by the reprotonation of Asp-96 from the medium (N → O, linearly dependent on the proton concentration). Experimental evidence that reprotonation occurs during the decay of N was given earlier by Skulachev et al. (1988), who referred to this intermediate as P. It is bothersome, however, that in the latter work the

M decay did not contain a slower component containing the time constant of N, predicted by the reversibility of the M ↔ N reaction, even though the relaxation kinetics of M and N were well separated. Inasmuch as having determined the kinetics of N and the reprotonation of BR under the conditions where the newly proposed kinetic model (Váró & Lanyi, 1990; Otto et al., 1989) applies, the present work provides additional information on the reprotonation question. The results were unambiguous and confirmed the earlier findings and models: the time course of the reprotonation of BR followed M *plus* N rather than M. This was particularly evident at pH 8.5 where the two alternatives predicted very different results (Figure 9). Thus, N must contain a deprotonated residue (i.e., Asp-96).

In the presence of azide, a mobile proton donor (Tittor et al., 1989), BR in which Asp-96 is replaced with asparagine will accumulate a large amount of an N-like intermediate after photoexcitation.[2] This species arises as M decays, together with O, and its spectrum is very similar to that suggested for N in wild-type BR. From the results, it would seem that the chromophore in N is rather indifferent to the state of Asp-96, and is defined instead by the isomeric state of the retinal, which is C=N anti, C_{13}-cis (Fodor et al., 1988). If the chromophore in N depends mainly on the retinal configuration, it is not clear why its lifetime should depend on the reprotonation of Asp-96. Thus, the pH dependency of the N decay (Kouyama & Nasuda-Kouyama, 1989; Váró & Lanyi, 1990) raises the interesting possibility that the isomerization around the C13–C14 bond during the N decay might be triggered by the reprotonation of Asp-96. A possible mechanism for this is suggested by calculations of Tavan et al. (1985), which state that the barrier to C13–C14 bond isomerization will be significantly lowered by removal of the influence of a negative charge on a protonated Schiff base. This charge could be Asp-96 itself, but its distance from the Schiff base seems too large at some 12 Å (Henderson et al., 1990).

We fully confirmed earlier results and suggestions at pH 7 (Drachev et al., 1984; Grzesiek & Dencher, 1986), that, when extrapolated to high proton conductivities, the proton release approached the rate of the rise of M, and the reprotonation of the BR correlated with the slow component in the decay of M (i.e., with the N relaxation, as it is now known). The stoichiometry appeared to be less than 1 H/BR, but the deviation from 1 was due to a kinetic artifact caused by the slowness of the dye response to pH change. It is evident from the data, however, that this information is not sufficient to describe the system. The time-resolved pH measurements showed more complex protonation behavior below pH 7 (Figure 4). As the pH was lowered, the magnitude and time dependency of the transient pH changed progressively, consistently with the results of independent measurements of photostationary states (Figure 1). All the features of the protonation kinetics could be accounted for by the sum of three effects: (1) the pH-independent release of a proton during the L → M reaction and the uptake of a proton during the decay of N. These are undoubtedly the steps comprising the vectorial proton transfer (cf. the introduction), since transport is pH independent between pH 4 and 9, as measured either by the rate of appearance of protons (Kouyama & Nasuda-Kouyama, 1989; Váró et al., 1990) or by photocurrents (Ormos et al., 1985). (2) The second effect is the pH-dependent uptake of a proton during the rise of M and its release during the decay of N, i.e., in a process occurring concurrently with the proton release and uptake in (1). We suggest that this occurs because in M and N the pK_a of a residue is raised to 5.9. The residue might be Asp-212, whose protonation during the L → M reaction was suggested by Braiman et al. (1988), although not by Gerwert et al. (1989). Alternatively, if Asp-212 does protonate at this step, it might be protonated intramolecularly, by that tyrosine whose deprotonation at this step was long proposed (Bogomolni et al., 1978; Hess & Kuschmitz, 1979; Hanomoto et al., 1984). (3) The third effect is the cooperative pH-dependent uptake of two protons in the O state. The pK_a of the two protonable residues in the O state appears to be 6.5. One of these residues may be the same as the residue in (2); in this case, that group remains protonated throughout the M, N, and O states, and O will have gained only one extra proton. Given the pH independence of proton transport, the participation of these residues in the transport is uncertain. It is more likely that their transient protonation is not vectorial (i.e., these are Bohr protons), and the shifts in their pK_a's reflect conformational changes and/or protonation changes in neighboring residues as BR passes through the photocycle.

Our earlier proposed model of the late events in the photocycle (Váró et al., 1990) and the present results raise some questions about the nature of O, and its role in the recovery of BR. A red shift of as much as 70 nm in the absorption maximum of this intermediate suggests that the interaction between the protonated Schiff base and its counterion must be intensely perturbed [for a recent discussion of such effects, see Baasov et al., (1987)]. A reasonable model for how this might happen uses the idea that Asp-212 and/or Asp-85 may be the counterion (or part of the counterion) for the Schiff base when the retinal is in the all-trans but not when it is in the 13-cis configuration. Thus, if Asp-212 and/or Asp-85 is protonated, the Schiff base in N is unaffected but in the next state, produced by reisomerization of the retinal, the Schiff base lacks a counterion. In this model, whether or not the pigment passes through O, and for how long, will depend on the relative rates of the isomerization and protonation reactions. In addition, in this model, O will be stabilized at low pH, as indeed observed (Li et al., 1984; Váró et al., 1990). We suggested that at low pH the direct, but normally slower, N → BR rate dominates (Váró et al., 1990). Both routes of N to BR, however, via O and directly, were compatible with proton transport.

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