# Photoreaction of the N Intermediate of Bacteriorhodopsin, and Its Relationship to the Decay Kinetics of the M Intermediate†

Leonid S. Brown,‡ László Zimányi,‡,§ Richard Needleman,‖ Michael Ottolenghi,⊥ and Janos K. Lanyi*,‡

*Department of Physiology and Biophysics, University of California, Irvine, California 92717, Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201, and Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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ABSTRACT: Because the M photointermediate of recombinant T46V bacteriorhodopsin decays more rapidly and the N intermediate more slowly than in wild-type, the photoreaction of N could be examined without interference from M. We found that between pH 6 and 9 the photoproducts of N included *both* earlier suggested M-like intermediate and red-shifted R state. However, when the photoexcitation of N was at wavelengths below 500 nm the amount of M-like product decreased with increasing pH, and at pH 9 virtually only R was produced. In the dark, T46V contains an N-like conformer, in increasing amounts with increasing pH like wild-type but in 4–5 times greater concentrations. The photoreaction of this thermally produced state is much like that of the N intermediate. It is associated with the appearance of a slowly decaying M, but we calculate that under most conditions used to follow M in the wild-type photocycle the amount of N-like conformer, and therefore the amplitude of this slow component, will not be significant. The results confirm the suggestion [Fukuda & Kouyama (1992) *Biochemistry 31*, 11740–11747] that an M-like state is included among the photoproducts of N, but at the same time provide support to photocycle models in which the slow component of the biphasic M decay is attributed not to this secondary photoreaction or to a separate photocycle originating from a heterogeneous initial state, but to thermal equilibration between M and N in a single photocycle.

Illumination of the light-driven proton pump bacteriorhodopsin (BR)[1] initiates a complex cyclic reaction of the retinal chromophore and the translocation of a proton from one membrane side to the other. The various states of the chromophore which arise and decay during this reaction cycle are spectroscopically distinguishable; they are referred to as J, K, L, M, N, and O [reviewed in Stoeckenius et al. (1979), Mathies et al. (1991), and Ebrey (1993)]. The exact description of the reactions in this "photocycle" has been uncertain because several of the proposed alternative schemes are kinetically equivalent, and evidence exists in favor of each of them. In particular, at pH >8 the decay of the M intermediate is approximated by two exponentials with widely different time constants. In the first of two kinds of proposed models, this originates from the development of a thermal equilibrium between M and the next intermediate N, and the subsequent slow decay of N (Otto et al., 1989; Váró & Lanyi, 1990b, 1991; Ames & Mathies, 1990; Gerwert et al., 1990; Souvignier & Gerwert, 1992). The model with the N to M thermal back-reaction accounts for the good correlation of the pH dependence of the time constants of the second decay component of M and the decay of N. Indeed, recent studies in which the kinetics of M are perturbed with a second flash in the blue or near-UV strongly suggest the existence of an N to M back-reaction (Druckmann et al., 1993; Zimányi et al., 1993). In the second kind of model, the biphasic M decay reflects two physically separate relaxation pathways. The slow M decay is proposed to arise either from secondary photoreaction of the N intermediate due to the measuring light (Kouyama et al., 1988; Fukuda & Kouyama, 1992) or from a minor subpopulation of the initial BR state which produces a differently decaying M intermediate (Kouyama et al., 1988; Tokaji & Dancsházy, 1992b; Fukuda & Kouyama, 1992). In this kind of model, the slow M decay component is regarded either as an artifact of the measurement or a complication by a BR conformer without intrinsic interest. Background illumination at high pH will indeed increase the amplitude of the slower M decay component (Fukuda & Kouyama, 1992), and the chromophore spectrum changes with increasing pH in a way which suggests the presence of an N-like conformer (Balashov et al., 1991; Fukuda & Kouyama, 1992). On the other hand, the detected photoproduct of N was described in several reports (Váró & Lanyi, 1990b; Balashov et al., 1990; Yamamoto et al., 1992; Tokaji & Dancsházy, 1992b) as red-shifted rather than blue-shifted like M. Some aspects of this problem are discussed in Zimányi et al. (1993). However, until all the conflicts in these observations are resolved, any proposed photocycle scheme remains open to question. To a large degree the reason for the conflict is that interference by other photointermediates of BR which coexist with N, particularly M, precludes direct and rigorous study of the N photocycle. It is to be noted that because the N intermediate contains a 13-*cis*,15-*anti*- rather than a 13-*trans*,15-*anti*- or 13-*cis*,15-*syn*-retinal chromophore (Fodor et al., 1988), its photoreaction cannot be predicted

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* To whom correspondence should be addressed.

‡ Univesity of California.

§ Permanent address: Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary.

‖ Wayne State University School of Medicine.

⊥ The Hebrew University of Jerusalem.

[1] Abbreviations: Br, J, K, L, M, N, and O, bacteriorhodopsin and its photointermediates; Bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; T46V, recombinant bacteriorhodopsin in which threonine-46 is replaced with valine. $^{N}$M (Kouyama et al., 1988; Fukuda & Kouyama, 1992) and R (Yamamoto et al., 1992) denote proposed M-like and red-shifted photoproducts, respectively, of the N intermediate.

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from the photoreactions of either the well-described light-adapted or the dark-adapted BR states.

In this report, we further explore (a) the photoproducts of the N photointermediate, (b) the pH-dependent heterogeneity of the protein, and (c) the possibility that these contribute to the observed kinetics of M. We examine these questions in the recombinant T46V bacteriorhodopsin. Threonine-46 is near D96, which is about 10 Å from the retinal Schiff base (Henderson et al., 1990). Although the absorption spectrum of the chromophore in T46V is nearly the same as in wild-type, the kinetics of M and N are considerably perturbed (Marti et al., 1991; Rothschild et al., 1992; this work). The decay of M in T46V is faster than in wild-type ($\tau$ = 300 $\mu$s at pH 9), and the decay of N much slower ($\tau$ = 1000 ms at pH 9). The apparent reason is that the T46V residue substitution lowers the p$K_a$ of D96 and hinders its access to protons from the cytoplasmic surface. There is no slow M decay component with the time constant of the decay of N. Thus, the N state which accumulates after flash photoexcitation can be probed with a second flash after most of M has decayed, and without interference from a possible N to M thermal back-reaction. Increasing the pH in the dark causes the same kind of amplitude decrease and blue-shift in the absorption spectrum, i.e., heterogeneity of the chromophore arising from the presence of an N-like state, as in wild-type (Fukuda & Kouyama, 1992), but to a much greater extent. These properties make T46V much better suited than wild-type for studying the photoreactions of N and the high-pH-related N conformer, and how they might contribute to the observed M decay kinetics.

The results demonstrate that at pH 6 photoexcitation of N produces *both* the proposed M-like species (Fukuda & Kouyama, 1992) and the red-shifted R state detected before (Balashov et al., 1990; Yamamoto et al., 1992; Tokaji & Dancsházy, 1992b). At higher pH, e.g., 9, this is not so. While photoexcitation with a 580-nm flash (i.e., near the absorption maximum of N) produces both R and M-like state, photoexcitation with a flash at 406 nm produces R but far less of the M-like state than expected from the extinction of N and from the amount of R. Blue measuring light will therefore also not produce M from N under these conditions. The R state and a slowly decaying M-like species are produced also by photoreaction of the high-pH-related N-like conformer. Judged from its amplitude in T46V, the amount of this M will be negligible in wild-type. We conclude that under the usual conditions for following the biphasic decay kinetics of M in wild-type the photoreactions of N and the N-like conformer are not the origins of the slow decay component.

## MATERIALS AND METHODS

The *Halobacterium halobium* strain which contains recombinant T46V bacteriorhodopsin was constructed as described before (Ni et al., 1990; Needleman et al. 1991). This bacteriorhodopsin, as well as the wild-type, was isolated from *H. halobium* as purple membranes by a standard method (Oesterhelt & Stoeckenius, 1974). All spectroscopy was with light-adapted purple membranes encased in polyacrylamide gels and equilibrated with the desired buffer or salt solutions, as described before (Needleman et al., 1991).

The two-flash experiments were with right-angle geometry, where the first flash was from a frequency-doubled Ne-Yag laser (532 nm, 7-ns duration) at 90° to the measuring beam, while the second flash was from a nitrogen-pumped dye laser (at the indicated wavelengths, 0.5-ns duration) at 270°. In generating the second flash, a number of different dyes with

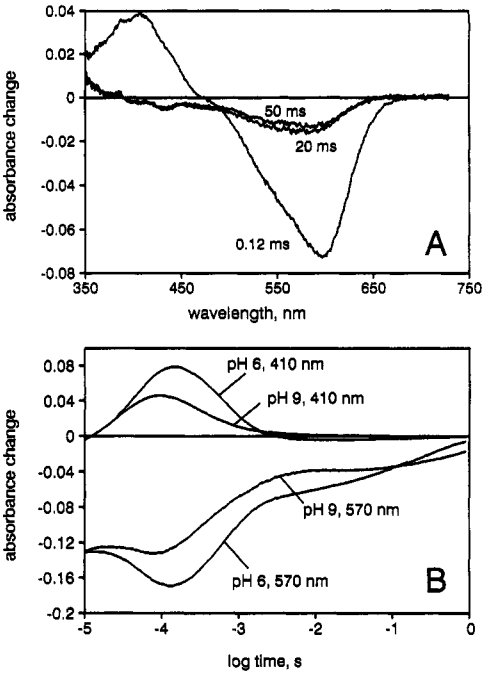


FIGURE 1: Transient difference spectra and kinetics in the photocycle of T46V bacteriorhodopsin. (*A*) Time-resolved difference spectra at pH 6, at 0.12, 20, and 50 ms after photoexcitation as indicated. (*B*) Absorbance changes at 410 and 570 nm as functions of time after photoexcitation, at pH 6 or 9 as indicated. Conditions: 25 $\mu$M T46V bacteriorhodopsin, 100 mM NaCl, 10 mM Bis-Tris propane, pH 6 or 9. Flash at 532 nm.

different wavelength maxima were used. Where the flash wavelengths were 580 or 406 nm, they are referred to as "yellow" and "blue" respectively. The intensities of these flashes were determined in separate measurements by the amount of wild-type bacteriorhodopsin they photolyzed. The instrumentation in these experiments was the same as described before (Váró & Lanyi, 1990a), except that the measuring light passed through an additional monochromator between the sample and the detector.

The instrumentation for optical multichannel spectroscopy was also as described before (Zimányi et al., 1989), but for photoexcitation the frequency-doubled Ne-Yag laser was used.

## RESULTS

*Photoreaction of the N Intermediate.* The photocycle of T46V bacteriorhodopsin is different from wild-type mainly in the rates of relaxations in the M to BR pathway (Marti et al., 1991; Rothschild et al., 1992). Figure 1A shows difference spectra at pH 6 at 120 $\mu$s, 20 ms, and 50 ms after a 532-nm flash, demonstrating the early decay of M which under these conditions leaves N as virtually the only intermediate after some tens of milliseconds. Figure 1B shows time courses of absorbance changes at 410 and 570 nm, at pH 6 and 9, respectively. The intensity of the monitoring light was low enough to not affect the kinetics. Likewise, the flash frequency used in signal averaging was low enough to allow completion of the photocycle. At pH 6 and 9, the time constants of M decay are 700 and 300 $\mu$s, respectively. The N decay is approximated by the much slower time constants of 350 and 1000 ms, respectively. The absorbance change at 410 nm is smaller at the higher pH because the faster M to N reaction results in more rapid and more complete conversion of M to N. As in Figure 1A, at pH 6 the absorbance change is consistent with complete decay of M in the 700-$\mu$s process. Closer examination of the difference between the 410-nm

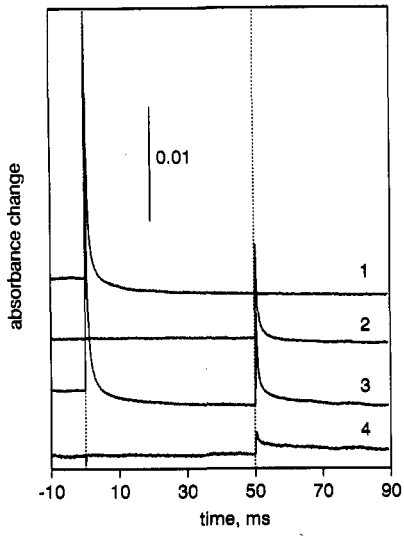


FIGURE 2: Determining the photoreaction of the N intermediate with a two-flash experiment. The first flash (532 nm) is at 0 time, the second (580 nm) at 50 ms. Absorbance was monitored at 425 nm. Trace 1, first flash only; trace 2, second flash only; trace 3, both flashes; trace 4, trace 3 *minus* trace 1 and trace 2 times 0.76 (the fraction of BR not photoexcited by the first flash). Conditions as in Figure 1, but at pH 9.

traces at pH 9 and 6 (Figure 1B) indicates, however, that a small amount of slowly decaying M ($\tau$ = approximately 100 ms) remains after the decay of the majority (cf. below). Its amplitude is very small, and if the spectra of M and N in this system are assumed to be like wild-type, it amounts to only about 3.5% of the photocycling fraction of BR. Thus, after tens of milliseconds, the N intermediate dominates in the photocycle even at pH 9: its concentration is about 30× the concentration of residual M. A secondary photoreaction induced after 20 ms will therefore have negligible contribution from M, and the N intermediate can be probed in this system with a second flash with minimal interference.

Figure 2 shows a two-flash experiment of this kind at pH 9; the absorbance changes were measured[2] at 425 nm which is near the maximum of M. The wavelength of the first flash was 532 nm; that of the second, provided after a delay of 50 ms, was 580 nm. Figure 2 shows records with the first flash alone (trace 1), the second flash alone (trace 2), and both flashes (trace 3). Subtraction of these two controls (trace from the second flash only multiplied by 1 *minus* the photocycling fraction of chromophore from the first flash, and trace from the first flash) yields the net effect of the second flash on the N state (trace 4). As shown in Figure 2, photoexcitation of N produces absorption changes which suggest a long-lived M-like state with complex decay kinetics. The two decay time constants are 1 and 60 ms; their amplitudes are about equal. Because the amount of BR photolyzed by the two flashes was greater than with the second flash alone, the possibility that the M decay kinetics might have been altered by a cooperative effect (Tokaji & Dancsházy, 1992a), and interfered with these measurements, was also considered. However, controls (not shown) indicated that in this system, and in the flash intensity range used, such effects are not significant.

The absorption changes were then determined after either a 580-nm (yellow) or a 406-nm (blue) second flash at 50 ms after the first flash. The intensity of the blue flashes was 1.7× greater than that of the yellow flashes. The absorbance

[2] This monitoring wavelength was chosen in order to minimize the flash artifact in experiments where 406-nm (blue) flash was used.

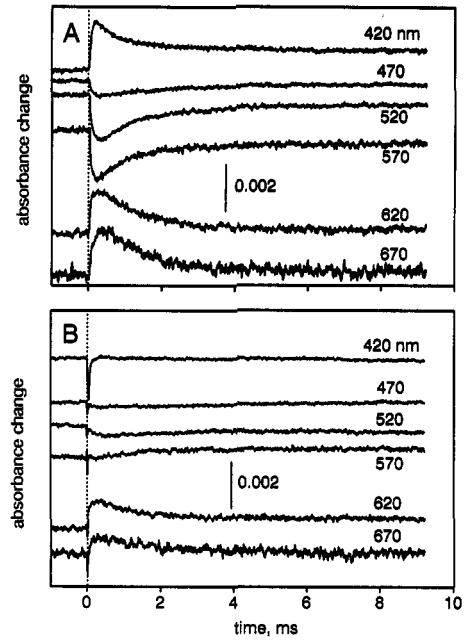


FIGURE 3: Absorbance changes from the photoreaction of N within 10 ms after a second flash. (*A*) Yellow second flash (580 nm): (*B*) blue second flash (406 nm). The experimental conditions were as in Figure 2. The traces shown correspond to the net absorbance change (trace 4) in Figure 2. The monitoring wavelengths are given in the figure.

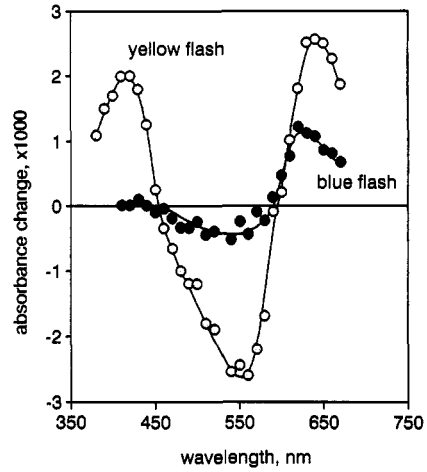


FIGURE 4: Maximal initial absorbance changes in the photoreaction of the N intermediate after yellow or blue second flashes, as functions of measuring wavelength. The maximal initial amplitudes (at 0.5 ms) in experiments such as shown in Figure 3 are plotted against the monitoring wavelength.

changes were followed at various monitoring wavelengths between 400 and 700 nm. Traces of the net absorbance changes within 10 ms of the second flash are shown in Figure 3A (yellow flash) and Figure 3B (blue flash). The wavelength dependencies of the amplitudes at 0.5 ms are given in Figure 4. With yellow flash, transient absorption increases are seen in both low- and high-wavelength regions, indicating that at least two photoproducts are present: one with a difference maximum at 420 nm (i.e., $^{N}$M), which arises with a time constant of 150 $\mu$s and decays with complex kinetics, and another with a difference maximum at 650 nm (i.e., R), which arises with a time constant of about 100 $\mu$s and decays with a time constant of 1.2 ms. *Unexpectedly, under these conditions, the blue flash yields a considerable amount of the red-shifted R state but virtually no absorbance change that would indicate that $^{N}$M is produced.* We had considered the possibility that under these conditions the absorbance

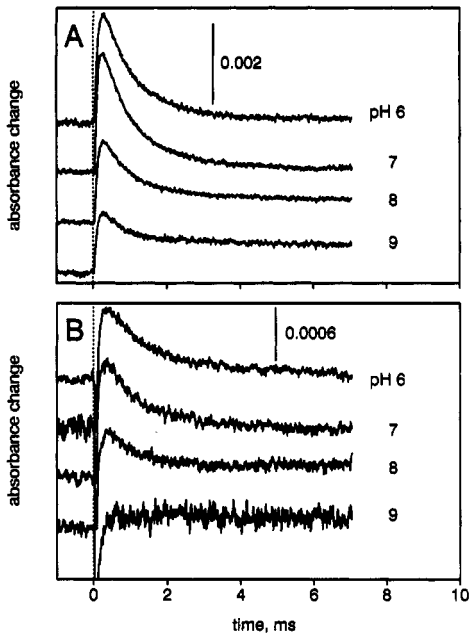


FIGURE 5: pH dependence of the photoreaction of the N intermediate measured at 425 nm. (*A*) Yellow second flash (580 nm); (*B*) blue second flash (406 nm). Experimental conditions as in Figure 3, but the second flash was at 20 ms after the first, and the pH was as indicated in the figure. Monitoring wavelength 425 nm.

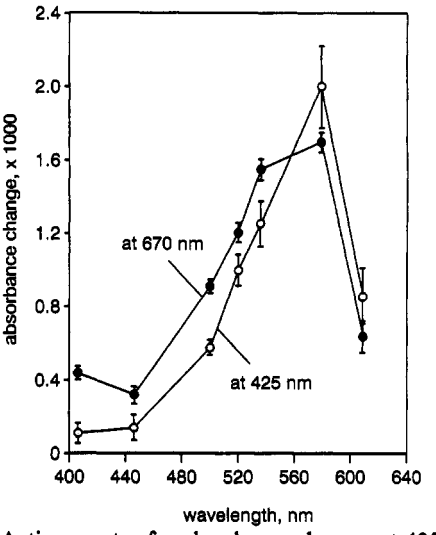


FIGURE 6: Action spectra for absorbance changes at 425 and 670 nm in the photoreaction of the N intermediate. Experimental conditions as in Figure 3, but the flash wavelength was varied as indicated. Monitoring wavelengths were either 425 nm (○) or 670 nm (●). Correction for variation of flash intensity with the different dyes used was made by measuring the amount of M photointermediate produced in a wild-type BR sample.

change might be absent because the blue flash depletes an amount of M just equal to that which is produced from N. In principle, a blue flash might deplete part of the small amount of M present at this time (Figure 1B), by a photoreaction described for M in the wild-type photocycle (Kalisky et al., 1978, 1981; Druckmann et al., 1993; Zimányi et al., 1993). However, we found that the small amount of residual M does not contribute to these effects. The absorbance change at 410 nm was not affected when the delay between the two flashes was increased to 550 ms (not shown), where M is virtually completely decayed but the majority of N is still present (the relevant decay time constants for M and N at this pH are 100 and 1000 ms).

We note that in Figure 3B the final levels of absorbance reached in the wavelength region between 570 and 620 nm appear to remain above the initial level. This was confirmed with 100-ms-long time scans (not shown), and indicates that the end product of this photoreaction absorbs more than the initial state. Thus, the photocycle which contains R appears to lead to BR rather than back to N.

The appearance of two distinct photoproducts raises the question that they might originate from different N substates. However, varying the delay time between the first and second (yellow) flashes between 20 and 1000 ms did not change the ratio of the observed amplitudes or the kinetics at 425 and 670 nm (at either pH 7 or pH 9, not shown); i.e., the same relative amounts of $^{N}$M and R were produced. Both amplitudes decreased with the amount of the N intermediate, as deduced from the absorption change at 570 nm after a single flash.

Figure 5A and Figure 5B show net traces measured at pH 6, 7, 8, and 9, at 425 nm after yellow and blue second flashes, respectively. The corresponding absorbance changes measured at 670 nm were all nearly pH-independent; they are not shown. It is evident from Figure 5A that with a yellow second flash the amplitude of $^{N}$M decreases somewhat with pH between 6 and 9, and this decrease reflects largely the smaller amplitude of the rapid decay component and the increased amplitude of a slower decay component. These amplitudes vary with pH somewhat similarly with blue second flash (Figure 5B), but as shown in Figures 3 and 4 already, at pH 9 little or no $^{N}$M is detected. Comparing the relative yields of R and $^{N}$M with yellow and blue second flashes revealed that less $^{N}$M is produced in the entire pH range with blue flash than expected from the photoreaction with yellow flash. For example, even at pH 6 where the amount of $^{N}$M is maximal, the amplitude ratio at 425 and 670 nm was 0.7 with blue flash and 1.6 with yellow flash. Quantitation of the results indicated that blue flash produces R in about the amount expected from the extinction of N near 400 nm relative to 570 nm, but depending on the pH much less or no $^{N}$M.

Action spectra for the production of $^{N}$M and R were determined at pH 9 with the second flashes set to different wavelengths. Figure 6 shows net amplitudes of the effect of second flashes 50 ms after an initial 532-nm flash, using seven different dyes which cover the wavelength region from 400 to 610 nm. Much smaller amounts of photoproduct are detected at 425 nm ($^{N}$M) than at 670 nm (R) when the second flash is in the blue region, but the reverse is true in the red region, confirming the results in Figures 3, 4, and 5. The shapes of the two action spectra in Figure 6 are approximately what one would expect for N. They suggest although do not necessarily prove that $^{N}$M and R originate from different photoactive N states with somewhat shifted spectra.

Although more difficult, the effect of changing the wavelength of the second flash was determined in a separate study (Druckmann et al., 1993) also for wild-type bacteriorhodopsin. Here the second flash depletes M, and its subsequent partial recovery was studied to decide if it originates from a thermal N to M back-reaction or the photoreaction of N. The amplitude of M recovery relative to M depletion was found to be wavelength-independent between 337 and 470 nm, arguing that M is not produced by an N photoreaction in the wild-type system either.

*Photoreaction of the N-like Conformer at High pH.* Small shifts and amplitude changes in the spectrum of unphotolyzed wild-type bacteriorhodopsin with increasing pH above 8 suggest some pH-dependent heterogeneity. At high pH, there are at least three additional states distinguishable by their contributions to high-pH *minus* low-pH difference spectra:

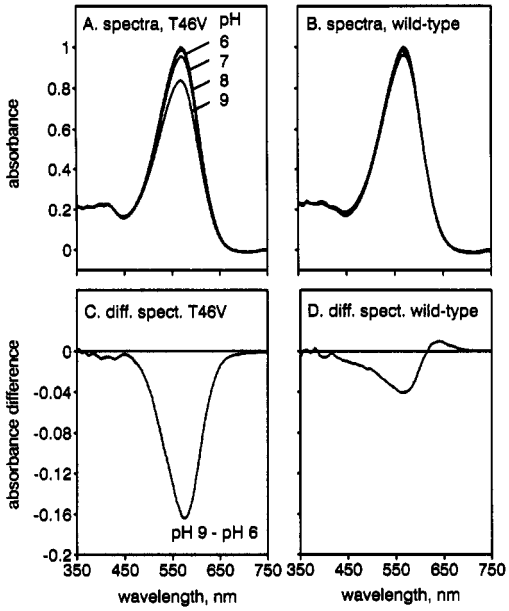


FIGURE 7: Spectra of T46V and wild-type bacteriorhodopsins, and their dependence on pH. Spectra (*A* and *B*) were measured for light-adapted samples in 2 M NaCl/10 mM Bis-Tris propane at the pH indicated. Panels *C* and *D* show the spectra at pH 9 *minus* the spectra at pH 6 for T46V and wild-type bacteriorhodopsins.

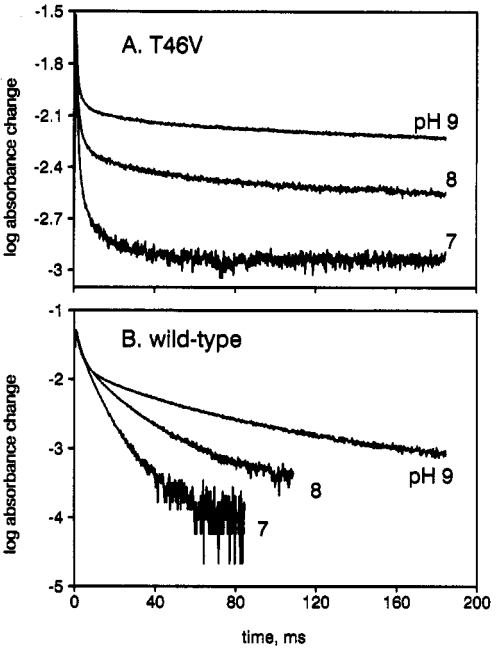


FIGURE 8: Decay kinetics of the M intermediate in T46V and wild-type bacteriorhodopsins, and their dependence on pH. Experimental conditions as in Figure 7. Flash wavelength, 532 nm; monitoring wavelength, 410 nm.

(a) a strongly blue-shifted species of unknown nature[3] termed P480 (Balashov et al., 1991); (b) an N-like species with lower amplitude and a somewhat blue-shifted maximum (Fukuda & Kouyama, 1992); and (c) a red-shifted species which gives a small difference maximum at about 640 nm, and is attributed to the effect of tyrosine ionization on the chromophore (Balashov et al., 1991). It was reported (Fukuda & Kouyama, 1992) that P480 is not produced below pH 10 at several molar salt. Figure 7A and Figure 7B show spectra of light-adapted T46V and wild-type bacteriorhodopsins, respectively, at pH 6, 7, 8, and 9 in the presence of 2 M NaCl so as to avoid the complication of P480. The presence of the high salt concentration has the additional consequence that the pH at the membrane surface will be higher than at lower salt concentrations and approach that in the bulk (Szundi & Stoeckenius, 1989), and thus effects caused by raising the pH will be enhanced. Figure 7A,B shows that the spectrum of light-adapted T46V at pH 6 is hardly perturbed: its maximum is at 571 nm, or about 2 nm red-shifted from wild-type. Raising the pH causes progressive changes in the spectrum. The differences between the spectra measured at pH 9 and 6 are given in Figure 7C,D. The absence of an absorption increase near 500 nm confirms that P480 is largely absent under these conditions. The difference spectrum for wild-type (Figure 7D) reflects both the N-like state and the red-shifted species; both are probably present in T46V as well (Figure 7C), but the positive feature in the red is masked by the large amplitude of the minimum. Other than the band near 640 nm, the difference spectra in the two systems resemble the difference spectrum for N (Dancsházy et al., 1988; Fukuda & Kouyama, 1992; Figure 1A). The red-shifted minimum of the difference spectrum in Figure 7C (at 575 nm) indicates that the N-like conformer of T46V absorbs somewhat to the blue of the main BR band as in wild-type (Kouyama et al., 1988). Because the difference spectrum for the thermally produced N-like state at the higher pH is quite different from those obtained between dark-adapted and light-adapted bacteriorhodopsin [e.g., see Bogomolni et al., (1980)], it is unlikely to contain the 13-cis,15-syn configuration of the dark-adapted chromophore. The N-like state produced under these conditions thus resembles more the N state in the photocycle. The amplitude of these changes is considerably greater in T46V than in wild-type at the same pH. If the N-like conformer of T46V has the same spectrum as the corresponding state in wild-type, its amount is therefore much greater. For example, at pH 9, T46V appears to contain 4–5 times more N-like conformer than the wild-type (Figure 7C,D).

Figure 8A and Figure 8B show M decay in T46V and wild-type, respectively, measured at 410 nm at pH 7, 8, and 9 under the same conditions as the spectra in Figure 7. As in Figure 1, care was taken to use measuring light intensities (410 nm) low enough not to have an effect on the absorption changes, and to signal-average at frequencies low enough to allow the virtually full recovery of the initial BR state. In T46V, a slowly decaying M state appears with an amplitude increasing with pH (cf. also Figure 1B). Its decay time constant at pH 9 and 2 M NaCl is 860 ms, while N decays under these conditions with a time constant of 1400 ms. The amplitude of the slow M decay component in T46V is about 3% of the total at pH 7, 8% at pH 8, and 20% at pH 9 (Figure 8A). This is similar to the way the absorbance change changed with pH in Figure 7A: the decrease at pH 7 was one-eighth of the decrease at pH 9 and one-third at pH 8. As suggested for wild-type (Fukuda & Kouyama, 1992), there seems to be a correlation between the pH-dependent changes in the spectrum of the protein and the appearance of a slowly decaying M. The concentration of this M at pH 9 corresponds to about 5% of the photocycling amount of BR. Absorbance changes at 670 nm (not shown) indicated that R is also produced, with increasing amplitudes as the pH is raised from 6 to 9. Its rise and decay kinetics were similar to the R state produced from the N intermediate (Figure 3), and distinguished it from an O state which would also absorb in the red but appears to be absent in this system at pH ≥6.

[3] When produced by adding dimethyl sulfoxide, P480 contains an all-trans chromophore (Pande et al., 1989). The blue shift of its maximum is attributed in this case to small changes in the charge environment of the Schiff base.

The flash wavelength in Figure 8 was 532 nm; we had tested also the effect of flashes at 406 nm. Consistent with the photoreactions of the N intermediate of T46V at 100 mM NaCl and pH 9, when the blue flashes were used the amplitude of the slowly decaying M was so low as to be undetectable (not shown).

A slowly decaying M appears in wild-type at higher pH also, but with more complex kinetics (Figure 8B). At pH 9, its amplitude is 27% of the total amount of M, or 22% of the photocycling amount of BR. This is much more than in T46V, in spite of the 4–5× smaller absorption changes in Figure 7B. Since it appears that all of the slowly decaying M of T46V originates from the photoreaction of the N-like conformer, the relative amplitudes of the difference spectra in Figure 7C,D indicate that in wild-type the amount of slowly decaying M is expected to be less than 1% of the photocycling fraction. Thus, in wild-type only an insignificant part of the observed slow decay component of M will be related to this kind of heterogeneity. Consistent with this conclusion and in contrast to the observations with T46V (cf. above), the biphasic decay of M in wild-type was about the same when the flash wavelength was set to either 532 nm or 406 nm (not shown).

## DISCUSSION

We have examined both proposed mechanisms by which M might be produced by a photoreaction of N. The first is the photoreaction of the N intermediate, the second is the photoreaction of an N-like conformer present at high pH in the unphotolyzed protein. We studied these effects in the T46V residue-substituted bacteriorhodopsin. If the chromophore in this recombinant protein is essentially unperturbed, as we believe on the basis of the considerable distance of T46 from the retinal in the structure (Henderson et al., 1990) and the absorption spectrum which is like wild-type (Figure 7), the results settle the question of the photoreaction of N in the wild-type system also. This conclusion is supported by results with the wild-type protein (Druckmann et al., 1993).

We find that when the photoexcitation of the N intermediate is in its main absorption band, i.e., with a yellow flash, both the earlier proposed $^{N}$M (Kouyama et al., 1988; Fukuda & Kouyama, 1992) and R (Balashov et al., 1990; Yamamoto et al., 1992; Tokaji & Dancsházy, 1992b) are produced, and with similar spectral amplitudes (Figures 3A and 4). The relationship of these to one another is not clear. Their kinetics do not readily suggest how they might fit into a photocycle scheme. If they originate from two distinct N intermediates (Figure 6), the putative N substates are not temporally separated in the photocycle, although they might be in a pH-dependent equilibrium (Figure 5). It is therefore possible that either N itself or the first stable state in the N photocycle is heterogeneous and there exist two distinct pathways (i.e., "N photocycles") for the thermal transformations which follow. It appears from the data that the photocycle which contains R leads to BR rather than back to N, i.e., results in a 13-cis,15-anti to 13-trans,15-anti transformation. Such a reaction was suggested on the basis of increased proton transport turnover rates at high light intensities and high pH (Kouyama & Nasuda-Kouyama, 1989). The dependence of the amplitude of the slowly decaying form of $^{N}$M on pH suggests that it depends on the deprotonated state of a residue. This residue might be D96. Replacement of D96 with asparagine or alanine gives a similarly pH-dependent slowed M decay, although with considerably greater time constants (Tittor et al., 1989; Holz et al., 1989; Miller & Oesterhelt, 1990; Cao et al., 1991). A lowered p$K_a$ for D96 in T46V relative to wild-type is suggested also by the changed kinetics of the M in the T46V photocycle (Marti et al., 1991).

When N is excited at pH 6 near its minor absorption bands at about 400 nm, i.e., with a blue flash, many of the same photoproducts are seen as with a yellow flash although with less apparent quantum yield. Unexpectedly, however, at pH 9 R is produced, but little or no $^{N}$M is produced. The reason for this is not clear. The absorption amplitude in the blue wavelength region is roughly the same, relative to the amplitude of the main band, in all states with a protonated Schiff base. This is true for N as well, although the N *minus* BR difference spectrum in Figure 1A (20- and 50-ms spectra) in this region is structured and slightly below zero. Photoexcitation in the blue wavelength region generates retinal excited states of higher energy than the lowest $^{1}B_{u}^{*+}$ singlet state [reviewed in Birge (1981)]. In BR, the higher excited states lead to the same bond rotations which are associated with isomerization from 13-trans,15-anti to 13-cis,15-anti as the lowest state. However, in N, where the retinal is in the 13-cis,15-anti configuration, the shape of the potential surfaces of the excited states must be different than in BR. Evidently, the higher excited states either of a single N state or of those of one of several N states lead to a different mixture of initial photointermediates than the lowest excited state. While the initial events responsible for the lack of an M-like photoproduct under these conditions are uncertain, these findings have important implications for interpreting the M decay in the BR photocycle: *When the M state is followed at high pH, the blue monitoring light will not contribute directly to the measured M kinetics through photoexcitation of N.* Erroneous M decay under these conditions will be observed only if N accumulates in a photostationary state either because the monitoring light is excessively intense or because the (yellow) flash frequency is too high relative to the turnover of the the photocycle. In either case, M will be produced from the N photoexcited together with BR. In the studies reported here, we avoided such conditions.

The results with T46V also address the question of the relationship between M decay and the pH-dependent heterogeneity in unphotolyzed bacteriorhodopsin. Absorption changes with increasing pH, which indicate thermal equilibrium of BR and an N-like conformer, occur both in wild-type and in T46V, but with 4–5 times smaller amplitude in the wild-type (Figure 7). The thermal stability of this N-like state is surprising in view of the fact that in this pH range the N photointermediate decays rather completely to BR. Another example of a small change in the protein which stabilizes states resembling photocycle intermediates is the case of D85N bacteriorhodopsin. In this system, the unphotolyzed chromophore appears to be a pH-dependent equilibrium mixture of M-like, N-like, and O-like states (Turner et al., 1993).

In T46V, the absorption changes from what appears to be a thermal 13-cis,15-anti state at high pH and high ionic strength (Figure 7A) correlate with the appearance of slowly decaying M (Figure 8A). The corresponding absorption changes in wild-type are 4–5 times smaller (Figure 7C,D). This is consistent with the fact that at pH 9.5 in the presence of 3 M KCl the amount of thermally arising 13-cis chromophore was too small to be detected by resonance Raman (Fodor et al., 1988). One would expect that the slowly decaying component of M would be much less in wild-type than in T46V. The correlation of the amplitude of the absorption changes and the amplitude of the slowly decaying M predicts that at high pH, and particularly in the presence of molar salt concentrations, the N-like state will affect the

observed M decay kinetics in T46V but not contribute significantly to the M decay kinetics in wild-type. We conclude from these results that under the usual conditions for studying M *the origin of the slow M decay component in the wild-type photocycle is not photoexcitation of the pH-dependent N-like conformer.*

The results thus support photocycle models which attribute the biphasic M decay kinetics not to the photoreaction of N or the N-like conformer but to a thermal back-reaction from N. The implications of this kinetic feature for the proton affinities in the D96 to Schiff base proton transfer are discussed elsewhere (Váró & Lanyi, 1991; Cao et al., 1991; Lanyi, 1992).

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