# Effects of Tyrosine-26 and Tyrosine-64 Nitration on the Photoreactions of Bacteriorhodopsin†

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ABSTRACT: In the dark, all titratable tyrosine residues of bacteriorhodopsin have p*K*'s of >11.0, which may be caused by the hydrophobic environment for buried residues and by high negative charge density for surface residues [Scherrer, P., & Stoeckenius, W. (1984) *Biochemistry 23*, 6195–6202]. Under illumination, deprotonation of only one tyrosine is observed in the micro- and millisecond time ranges of the photocycle; this is Tyr-64. Nitration of Tyr-64 decreases the chromophore absorbance, shifts the absorption maximum to 535 nm, and affects photocycle kinetics. However, restoring its native p*K* by reduction after nitration has no effect on the changes in photocycle kinetics or absorbance of the chromophore. Nitration of Tyr-64 apparently causes a conformational change in bR, which is independent of the p*K* of its phenolic group. These observations contradict earlier conclusions that in the photocycle a tyrosine residue directly interacts with the Schiff base during its deprotonation or reprotonation. The protonation state of Tyr-26 and the alkaline chromophore transition are correlated, as shown earlier (Scherrer & Stoeckenius, 1984). Lowering the p*K* of Tyr-26 by nitration decreases the M-decay rate, and this effect is partially reversed by reduction of the nitro group. We conclude that Tyr-26 may be located close to the chromophore and interact with it; but its protonation state does not change at physiological pH and in the microsecond time range of the photocycle. Tyr-64 is apparently located at or close to the external surface; its modification strongly affects the chromophore but apparently indirectly and not through its protonation changes. Neither nitration nor amination of Tyr-64 or Tyr-26 eliminates light-driven proton translocation in reconstituted lipid vesicles.

Bacteriorhodopsin (bR),[1] the only protein in the purple membrane (pm) of *Halobacterium halobium*, functions as a light-driven proton pump (for review, see Stoeckenius et al. (1979) and Stoeckenius & Bogomolni (1982)]. Its primary sequence is known (Ovchinnikov et al., 1979; Khorana et al., 1979), and the polypeptide chain apparently traverses the membrane with seven $\alpha$-helical segments (Khorana et al., 1979; Engelman et al., 1980; Agard & Stroud, 1982). Like visual pigments, bR contains a retinylidene chromophore (Oesterhelt & Stoeckenius, 1971), and its retinal–Schiff-base linkage to lysine-216 is protonated (Lewis et al., 1974). When illuminated, the chromophore undergoes a cyclic photoreaction comprising at least four spectroscopically distinct intermediates (Lozier et al., 1975; Lozier & Niederberger, 1977). The photocycle is accompanied by a transient deprotonation of the Schiff base and a transient all-trans to 13-cis isomerization of the retinal (Lewis et al., 1974; Pettei et al., 1977; Braiman & Mathies, 1982).

The molecular mechanism of the light-driven proton translocation is not known. It apparently involves transient deprotonation of the Schiff-base linkage, of tyrosine(s) (Bogomolni et al., 1978; Hess & Kuschmitz, 1979; Scherrer et al., 1981; Lemke et al., 1982; Hanamoto et al., 1984), and of carboxyl groups (Rothschild et al., 1981; Engelhard et al., 1985). Selective chemical modification of specific amino acids provides information on their function and the arrangement of the polypeptide chain. We have developed a method for the selective nitration of tyrosine-26 and -64 with tetranitromethane (Scherrer & Stoeckenius, 1984) and studied the effects on the chromophore absorbance. Here we describe the effects on late events in the photocycle and use the absorption bands at 360 and 428 nm of protonated and unprotonated nitrotyrosine (Riordan et al., 1967) to follow the protonation state of the nitrated tyrosine-64.

## Materials and Methods

*Materials.* Purple membrane from *H. halobium* (strain ET1001 provided by Dr. J. Weber) was isolated as described (Oesterhelt & Stoeckenius, 1974). The pm suspension was stored either at 4 °C in 2 M NaCl or at −80 °C in 40% aqueous sucrose solution.

*Selective nitration* of tyrosine-26 and tyrosine-64 in pm and their reduction with sodium dithionite have been described (Scherrer & Stoeckenius, 1984). The nitrated preparations will be called bR-N26 and bR-N64; subsequent reduction will be indicated by adding the letter R.

*Absorption spectra* of native and nitrated pm were recorded with an Aminco DW-2a spectrophotometer linked to a Nicolet 1074 data acquisition system. Concentrations of bR were determined spectroscopically with $\epsilon_{570nm}$ = 63 000 $M^{-1}$ $cm^{-1}$ and $M_r$ 26 000 for unmodified bR and bR-N26 and $\epsilon_{535nm}$ = 46 000 $M^{-1}$ $cm^{-1}$ for bR-N64 (Scherrer & Stoeckenius, 1984).

*Flash Spectroscopy.* Rapid absorbance changes were measured as described (Lozier, 1982). The pm samples were suspended in 10 mM NaCl (100–150 $\mu$g/mL) at 20 °C, and the pH was adjusted with HCl or NaOH. The 10-ns actinic

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[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; TNM, tetranitromethane; bR-N26, bacteriorhodopsin with nitrotyrosine in position 26; bR-N64, bacteriorhodopsin with nitrotyrosine in position 64; bR-N26R, bacteriorhodopsin with aminotyrosine in position 26; bR-N64R, bacteriorhodopsin with aminotyrosine in position 64; M-N26, photocycle intermediate M of bR-N26; O-N26, photocycle intermediate O of bR-N26; M-N64, photocycle intermediate M of bR-N64; O-N64, photocycle intermediate O of bR-N64; $\Delta E$, transition energy shift; HPLC, high-pressure liquid chromatography.

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light pulse at 520 nm, near the isosbestic point of bR and bR-N64, was provided by a nitrogen-pumped dye laser (Molectron, Sunnyvale, CA). The measuring and actinic beams were polarized, and all measurements were done with beam polarizations at magic angle. Kinetics of the formation and decay of the intermediates $M_{410}$ and $O_{640}$ were obtained as reported by Lozier & Niederberger (1977) and analyzed graphically.

*Light-driven proton translocation* activity was measured as described (Racker & Stoeckenius, 1974) with the following modifications; 10 mg of crude soybean phospholipids was first sonicated alone in 0.9 mL of 200 mM KCl–200 mM sucrose in a bath sonicator (Laboratory Supplies Co. Inc., New York) under nitrogen atmosphere for 5 min, and 0.1 mL of purple membrane suspension containing (500 $\mu$g) bR was then added to the transparent vesicle suspension, and the sonication was continued for 10 min. After the dilution to 1 $\mu$M bR, the light-induced pH changes were measured with a glass electrode immersed in 2 mL of vesicle suspension at pH 6.2–6.4 containing 1.9 nmol of bR, 200 mM KCl, and 200 mM sucrose in a conical glass cell with a surrounding water jacket thermostated at 25 °C. The mixture was stirred magnetically and illuminated with a 100-W halogen lamp through a heat filter and a 3-69 sharp-cut yellow filter (Corning) with a light intensity of $2.5 \times 10^{-5}$ erg cm$^{-2}$ s$^{-1}$. The system was calibrated with standard HCl additions at the end of each measurement. The activity of proton pumping was calculated from the initial rate of the pH change and the absorption of the vesicle suspension. The rates were measured at low light levels, where the rate of translocation increases linearly with the increase in light intensity.

*Extraction of Retinal.* A total of 0.2 mL of pm suspension containing 1–2 mg of bR/mL in 10 mM NaCl, pH 6.2, was mixed rapidly with 0.5 mL of ice-cold ethanol on a vortex mixer. After 2 min on ice, 0.5 mL of ice-cold hexane was added and the sample mixed repeatedly during 2 min. The suspension was centrifuged in a Beckman Microfuge, and the retinal isomers in the hexane phase (upper) were separated by HPLC (Spectra physics) on two Sorbex Sil columns (4.6 × 250 mm each; Du Pont) in series with 8% ether in hexane as solvent at a flow rate of 1.5 mL/min. The extraction was done under dim red light.

## Results

*Flash-Induced Difference Spectra of bR, bR-N26(R), and bR-N64(R).* The flash-induced difference spectra of bR-N26 and bR-N26R in the millisecond time range were within experimental errors identical with the unmodified bR spectra (data not shown). The difference spectra for bR and bR-N64 1 ms after the actinic flash (Figure 1) are also very similar except for a blue shift of the bR-N64 spectrum, resulting from the 30-nm blue-shifted chromophore absorbance maximum at 530–540 nm (Scherrer & Stoeckenius, 1984). The excitation light of 520 nm is near the isosbestic point of the bR and bR-N64 absorbance at 520–525 nm, and therefore, we expect to excite similar numbers of molecules in both preparations. The short-wavelength absorbance maximum due to intermediate M in bR is only about 10 nm blue-shifted in bR-N64, and its extinction coefficient $\epsilon_{400}$, calculated from the depletion at 540 nm [using $\epsilon_{540}$ = 46 000 M$^{-1}$ cm$^{-1}$ for bR-N64 (Scherrer & Stoeckenius, 1984)], is similar to the $\epsilon_{410}$ of M in native bR. The formation of intermediate O causes an absorbance increase at 660 nm in bR 15 ms after the flash (Figure 1). A similar absorbance increase occurs in bR-N64 at 620 nm and 75 ms after the flash. The calculated absorbance maximum for this O-like intermediate in

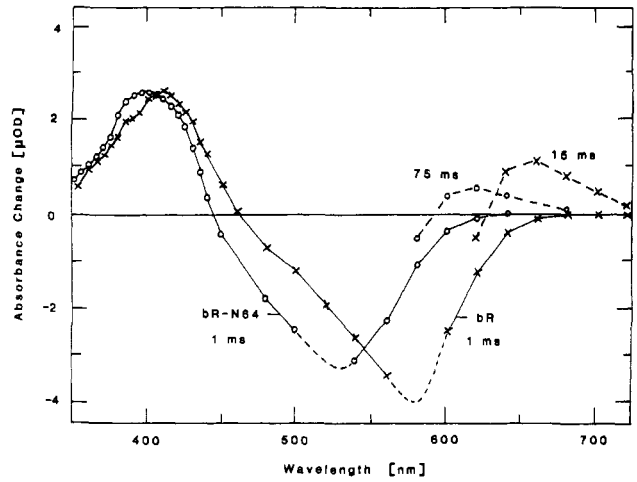


FIGURE 1: Absorbance changes of bR (×) and bR-N64 (○) in 10 mM NaCl, pH 7.0, at 1 ms (solid line) and 15 (bR) and 75 ms (bR-N64) after the actinic flash (broken line). The spectrum before the flash was subtracted from the spectra after the flash.

bR-N64 is at 600 nm, 40 nm blue-shifted from $O_{640}$ in bR. Changing the actinic wavelength from 520 to 500 or 580 nm had no effect on the shape of the transient difference spectra and the formation and decay kinetics of the intermediates. This indicates that the blue-shifted chromophore is cycling and that the preparation does not contain a mixture of several different, cycling chromophores. Because of the obvious correspondence of the M and O intermediates of bR to those of the nitrated preparations, we shall refer to the latter as M-N26, O-N26, M-N64, and O-N64, adding the letter R for reduced preparations.

The decreased effects of Tyr-64 nitration on the absorbance and blue shifts of the M-N64 intermediate compared to bR-N64 and O-N64 may be explained by the fact that the chromophore is unprotonated or that it is in the 13-cis configuration and presumably in a different environment less affected by the Tyr-64 modification. This latter explanation suggests that a similar effect may be observed in dark-adapted pm, which has at least 50% of its chromophores in the 13-cis configuration but with a protonated Schiff base.

*Light–Dark Adaptation of bR, bR-N26(R), and bR-N64-(R).* Whereas bR-N26 shows a dark adaptation indistinguishable from that of bR, the extent of the blue shift and absorbance decrease is much smaller in bR-N64 and bR-N64R (Figure 2). The spectra could be interpreted as a reduced extent of either light or dark adaptation. Extraction experiments show that dark-adapted bR-N64 contains a roughly equal mixture of 13-*cis*- and *all-trans*-retinal, which changes to >90% all-trans isomer upon exposure to light (Figure 2 insert); resonance Raman spectra are also consistent with a predominantly all-trans chromophore in light-adapted bR-N64 (S. Smith and R. Mathies, personal communication). The spectral change and extraction data for bR-N64R are indistinguishable from those of bR-N64. We conclude that the decreased influence of Tyr-64 nitration on the 13-cis isomer partly compensates for the decrease in absorbance and blue shift of the chromophore caused by the all-trans to 13-cis isomerization. The surprising observation that only ~5% of the total retinal are extracted from bR-N64 by techniques that extract 60–80% of the retinal from bR and bR-N26 preparations will be discussed later.

*Kinetics of the M and O Intermediates in bR-N64 and bR-N64R Compared to bR.* The traces for the transient absorbance changes of the M intermediates in all cases require two first-order components for a satisfactory fit. Their values

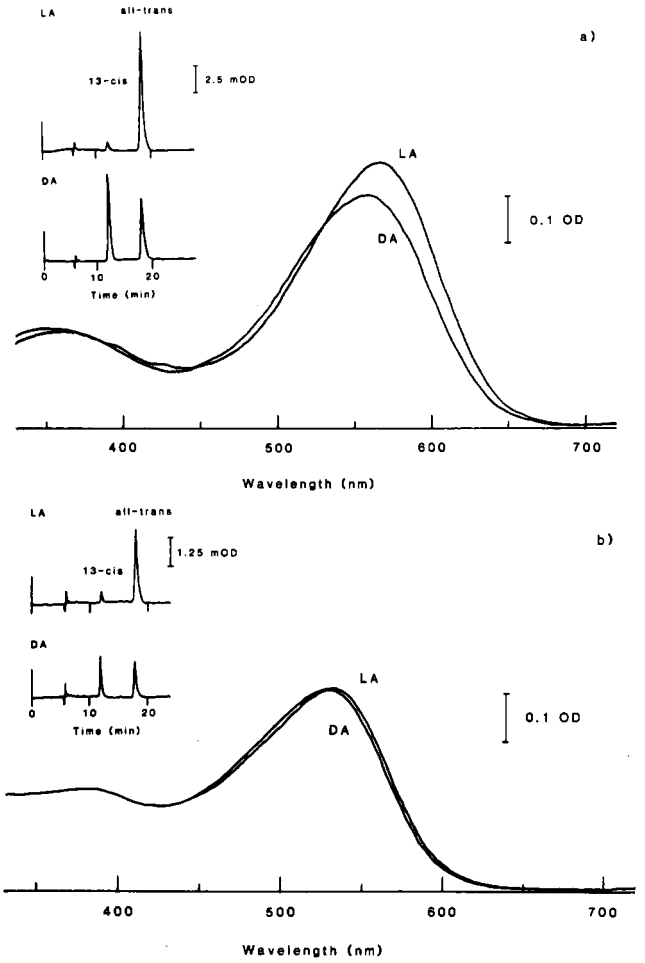


FIGURE 2: Absorption spectra of dark- and light-adapted bR-N26 (a) and bR-N64 (b) in 10 mM NaCl, pH 6.5 at 20 °C. The bR-N64R spectra (not shown) are indistinguishable from the bR-N64 spectra. The insert shows the HPLC elution profiles of retinal extracts from light-adapted and dark-adapted bR-N26 (a) and bR-N64 (b).

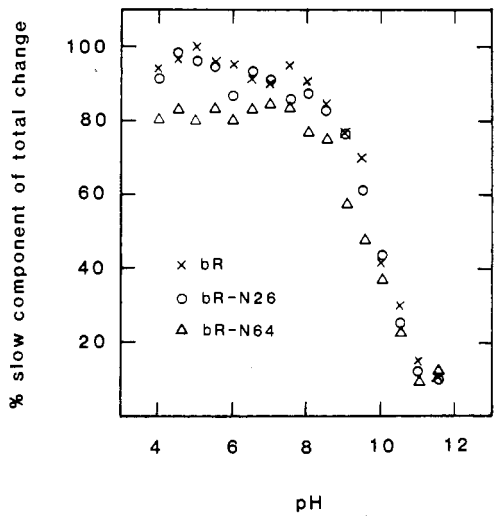


FIGURE 3: Titration curve of the slow absorbance component in $M_{400}$ formation in bR (×), bR-N26 (○), and bR-N64 (△). The total absorbance change is equal to 100% at any pH composed of a fast and slow component.

and the extents to which they contribute to the absorbance change vary with pH, and the extent of one may be so small that it is at the limit of detection. They will be identified by the subscripts f (fast) and s (slow). The O kinetics are satisfactorily described by one component in all cases. Large errors arise in the estimation of the components when their

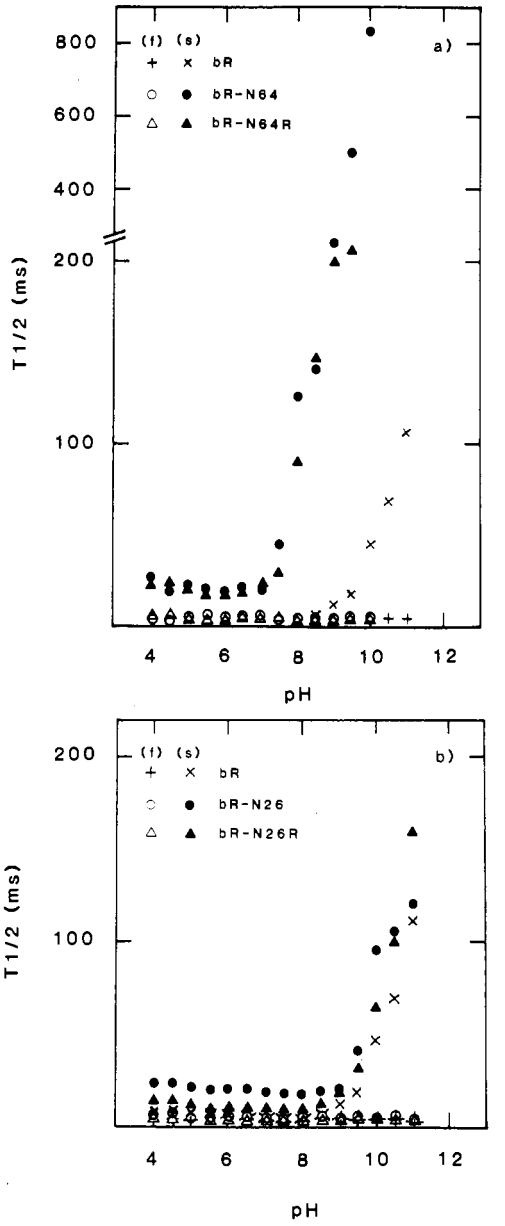


FIGURE 4: pH dependence of $\tau_{1/2}$ for M decay [fast (f) and slow (s) component] for (a) bR (+, ×), bR-N64 (○, ●), and bR-N64R (△, ▲) and (b) bR-N26 (○, ●) and bR-N26R (△, ▲).

contribution to the total absorbance change is small and/or their kinetics are not very different, and only differences by a factor of 1.5 or more can then be considered as significant. The data are summarized in Tables I–IV, and we shall describe here only the main features observed.

The rise of the fast component of the M and M-N64 absorbances is similar while the rise of the slow component of the M-N64 absorbance is slightly faster than that of M at all pH values. With increasing pH, the rates remain the same, but the faster component begins to contribute more in both cases and becomes dominant at pH >10 (Figure 3). The p$K$ of this transition is ~9.5 for M and M-N64. It is not changed by reduction.

The decay of M-N64 is slower than that of M. In both cases, its half-time increases at alkaline pH, but the steep increase begins at pH 7.5 for M-N64 and at pH 9.0 for M (Figure 4a). This is mainly due to the slower component. The extent to which it contributes to the total absorbance first decreases and at high pH increases again. The decrease in extent shows an apparent p$K$ of 6.5 for M-N64 and 8.5 for

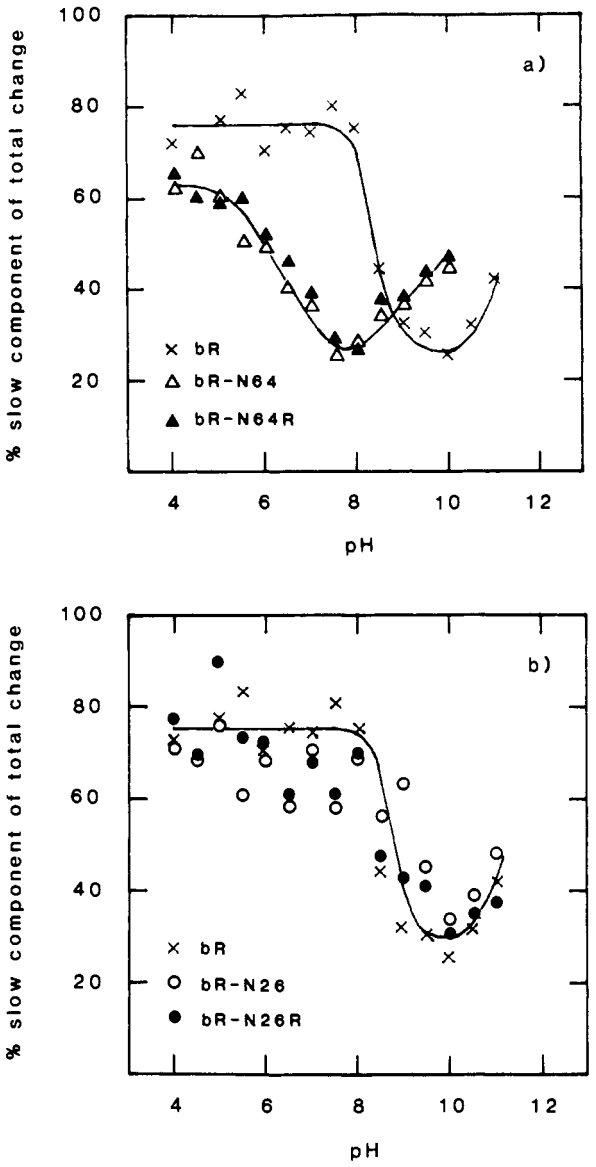


FIGURE 5: Titration curve of the slow absorbance component in M decay in 10 mM NaCl, at 20 °C, in (a) bR (×), bR-N64 (△), and bR-N64R (▲) and (b) bR (×), bR-N26 (○), and bR-N26R (●). The total absorbance change is equal to 100% at any pH composed of a fast and slow component.

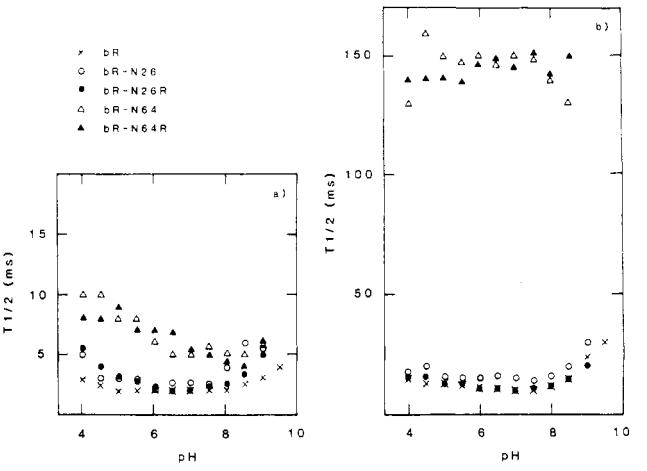


FIGURE 6: (a) pH dependence of $\tau_{1/2}$ for O formation for bR (×), bR-N26 (○), bR-N26R (●), bR-N64 (△), and bR-N64R (▲). (b) pH dependence of $\tau_{1/2}$ for O decay for bR (×), bR-N26 (○), bR-N26R (●), bR-N64 (△), and bR-N64R (▲).

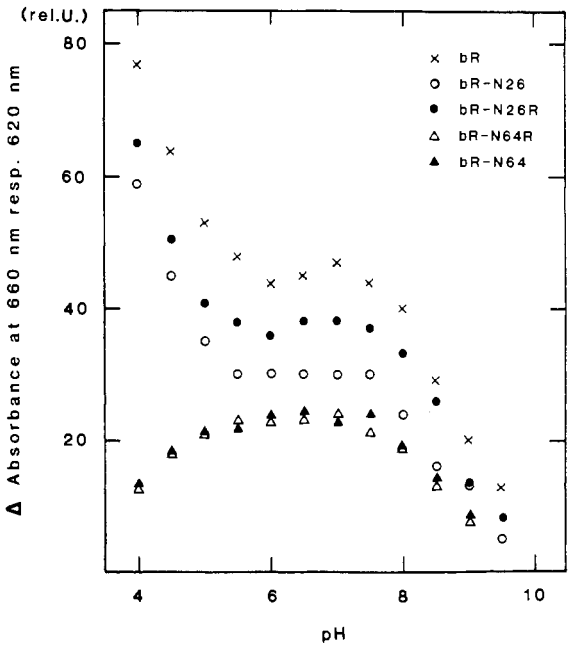


FIGURE 7: pH dependence of the absorbance increase (a) at 660 nm for bR (×), bR-N26 (○), and bR-N26R (●) and (b) at 620 nm for bR-N64 (△) and bR-N64R (▲).

M (Figure 5a). Again, reduction has no effect on the kinetics of the nitrated preparation.

The O rise time is 2–3 times slower in bR-N64 than in bR, whereas the O decay is 10 times slower, and neither is significantly affected by pH between 4.0 and 9.0 (Figure 6). The absorbance changes are satisfactorily fitted by a single-exponential component. However, the apparent O extent rapidly decreases in both preparations above pH 8.0, and at pH 9.0 it has practically disappeared (Figure 7). The extent is relatively constant between pH 8.0 and pH 5.0 and approximately 2 times higher for O than for O-N64. Some of the difference is probably due to a difference in the extinction coefficients, which we expect to be ~30% higher for O than for O-N64. Below pH 5.0, the apparent extent of O rises steeply, whereas that of O-N64 drops, but somewhat less rapidly. O-N64R shows the same behavior. The discrepancy in the pH dependence between native and nitrated preparation at low pH and the discrepancy between the kinetic changes and the extent at high pH suggest a branching of the photoreaction cycle at or before M and/or that at the wavelength of observation, 660 nm for bR and 620 nm for bR-N64, absorbance changes other than those due to O contribute. The possibility that rapid dark adaptation at low pH caused a contribution from intermediates of the 13-*cis*-bR cycle could not be substantiated; repeated light adaptation during data collection at low pH failed to reduce the apparent extent of O in native bR.

*Deprotonation of bR-N64 during the Photoreaction Cycle.* Nitrotyrosine has characteristic absorbance maxima at 360 nm in the undissociated form and at 428 nm in the ionized form with an isosbestic point at 380 nm (Sokolovsky et al., 1966). The extinction coefficients for the two forms ($\epsilon_{360}$ = 2200 $M^{-1}$ $cm^{-1}$; $\epsilon_{428}$ = 4100 $M^{-1}$ $cm^{-1}$) are small compared to the extinction coefficient of the M intermediate ($\epsilon_M$ ~ 42 000 $M^{-1}$ $cm^{-1}$), and protonation changes of nitrotyrosine during the photocycle are practically undetectable in the presence of the large absorbance changes caused by the M intermediate in this range. However, aminotyrosine lacks the absorption bands at 360 and 428 nm, and since we did not detect any differences in the kinetics and visible absorbance

Table I: Kinetics of M Formation Measured as the Absorbance Increase at 400 nm (Half-Times in Microseconds) at Various pHs in 10 mM NaCl at 20 °C[a]

| | bR | | | bR-N26 | | | bR-N26R | | | bR-N64 | | | bR-N64R | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | f | s | % | f | s | % | f | s | % | f | s | % | f | s | % |
| 4.0 | | 60 | 94 | | 58 | 91 | | 60 | 93 | | 45 | 80 | | 45 | 80 |
| 4.5 | | 58 | 97 | | 56 | 98 | | 63 | 89 | | 42 | 82 | | 40 | 84 |
| 5.0 | | 56 | 100 | | 58 | 96 | | 57 | 95 | | 38 | 80 | | 37 | 85 |
| 5.5 | | 58 | 96 | | 55 | 95 | | 58 | 92 | | 40 | 82 | | 45 | 74 |
| 6.0 | | 56 | 95 | | 60 | 86 | | 57 | 89 | | 38 | 79 | | 40 | 79 |
| 6.5 | | 58 | 92 | | 58 | 93 | | 60 | 88 | | 38 | 83 | | 40 | 76 |
| 7.0 | | 65 | 90 | | 65 | 90 | | 60 | 89 | | 40 | 84 | | 38 | 85 |
| 7.5 | | 62 | 95 | | 62 | 85 | | 64 | 92 | | 42 | 83 | | 42 | 83 |
| 8.0 | | 62 | 96 | | 64 | 87 | | 68 | 86 | | 45 | 76 | | 40 | 85 |
| 8.5 | | 64 | 85 | | 70 | 85 | | 67 | 84 | 6 | 46 | 74 | 6 | 42 | 78 |
| 9.0 | | 68 | 77 | 5 | 70 | 77 | 7 | 68 | 78 | 6 | 50 | 57 | 6 | 46 | 63 |
| 9.5 | 6 | 65 | 70 | 6 | 68 | 60 | 5 | 70 | 71 | 5 | 48 | 47 | 4 | 48 | 55 |
| 10.0 | 6 | 63 | 42 | 4 | 72 | 43 | 5 | 66 | 50 | 5 | 50 | 36 | 5 | 46 | 39 |
| 10.5 | 6 | 62 | 30 | 6 | 62 | 25 | 6 | 64 | 30 | 6 | 47 | 22 | 5 | 48 | 25 |
| 11.0 | 6 | 58 | 15 | 6 | 65 | 11 | 6 | 64 | 16 | 5 | 48 | 10 | 6 | 46 | 12 |
| 11.5 | 5 | 60 | 12 | | | | | | | | | | | | |

[a] Extent of contribution by the slow component is given as percent.

Table II: Kinetics of M Decay Measured as Absorbance Decrease at 400 nm (Half-Times in Milliseconds) in ~10 mM NaCl at 20 °C at Various pHs[a]

| | bR | | | bR-N26 | | | bR-N26R | | | bR-N64 | | | bR-N64R | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | f | s | % | f | s | % | f | s | % | f | s | % | f | s | % |
| 4.0 | 2.5 | 6.7 | 72 | 6.0 | 23 | 71 | 4.8 | 15 | 77 | 5.5 | 26 | 62 | 5.0 | 23 | 65 |
| 4.5 | | 6.8 | 88 | 6.5 | 23 | 68 | 3.5 | 16 | 69 | 4.0 | 20 | 70 | 5.0 | 24 | 60 |
| 5.0 | 3.0 | 7.0 | 77 | 5.0 | 21 | 76 | | 11 | 91 | 5.0 | 23 | 60 | 5.0 | 22 | 59 |
| 5.5 | 2.0 | 6.0 | 83 | 4.5 | 20 | 60 | 3.0 | 11.5 | 73 | 4.5 | 21 | 50 | 4.2 | 18 | 60 |
| 6.0 | 2.5 | 6.2 | 70 | 4.5 | 21 | 68 | 3.0 | 11 | 72 | 4.5 | 19 | 49 | 4.0 | 18 | 52 |
| 6.5 | 2.5 | 5.0 | 75 | 4.0 | 20 | 58 | 2.5 | 10.5 | 60 | 4.0 | 21 | 40 | 3.5 | 18 | 46 |
| 7.0 | 2.6 | 4.5 | 74 | 4.0 | 18 | 70 | 3.0 | 10 | 68 | 4.0 | 22 | 36 | 4.0 | 21 | 39 |
| 7.5 | 2.2 | 4.0 | 80 | 4.0 | 17 | 58 | 3.0 | 10 | 59 | 4.0 | 47 | 25 | 3.5 | 30 | 29 |
| 8.0 | 2.2 | 4.0 | 75 | 4.5 | 17 | 68 | 3.0 | 9.0 | 69 | 4.0 | 100 | 28 | 3.5 | 80 | 28 |
| 8.5 | 2.5 | 6.2 | 44 | 4.5 | 19 | 56 | 3.0 | 13 | 47 | 3.5 | 120 | 34 | 3.5 | 100 | 38 |
| 9.0 | 2.6 | 11.0 | 32 | 3.5 | 19 | 63 | 3.0 | 20 | 43 | 3.5 | ~220 | 37 | 3.5 | ~200 | 38 |
| 9.5 | 2.4 | 18 | 30 | 4.5 | 40 | 45 | 3.5 | 29 | 41 | 3.0 | ~500 | 42 | 3.0 | ~500 | 43 |
| 10.0 | 2.8 | 45 | 25 | 3.5 | 95 | 34 | 3.5 | 52 | 32 | 3.0 | ~900 | 44 | 3.0 | ~800 | 45 |
| 10.5 | 2.2 | 68 | 32 | 4.5 | 105 | 39 | 3.0 | 80 | 35 | | | | | | |
| 11.0 | 2.2 | 110 | 42 | 3.0 | 120 | 48 | 3.0 | 140 | 37 | | | | | | |

[a] Extent of contribution by slow component given as percent.

Table III: Kinetics of the Photocycle Intermediate *O Rise* Measured at 660 and at 620 nm for bR-N64(R) in Milliseconds at Various pHs in 10 mM NaCl at 20 °C

| pH | bR | bR-N26 | bR-N26R | bR-N64 | bR-N64R |
|---|---|---|---|---|---|
| 4.0 | 3 | 5 | 5.5 | ~10 | 8 |
| 4.5 | 2.5 | 3 | 4 | ~10 | 8 |
| 5.0 | 2 | 3 | 3 | 8 | 8–10 |
| 5.5 | 2 | 3 | 3 | 8 | 6–8 |
| 6.0 | 2 | 2 | 2 | 6 | 6–8 |
| 6.5 | 2 | 2.5 | 2 | 4–6 | 6–8 |
| 7.0 | 2 | 2.5 | 2 | 4–6 | 4–6 |
| 7.5 | 2 | 2.5 | 2.5 | 4–6 | 4–6 |
| 8.0 | 2 | 4 | 2.5 | 4–6 | 4 |
| 8.5 | 2.5 | 6 | 3.5 | 4–6 | 4 |
| 9.0 | 3 | 5.5 | 5 | 6 | 6 |
| 9.5 | 4 | | | | |

Table IV: Kinetics of the Photocycle Intermediate *O Decay* Measured at 660 and at 620 nm for bR-N64(R) in Milliseconds at Various pHs in 10 mM NaCl at 20 °C

| pH | bR | bR-N26 | bR-N26R | bR-N64 | bR-N64R |
|---|---|---|---|---|---|
| 4.0 | 15 | 18 | 16 | 130 | 140 |
| 4.5 | 13 | 20 | 16 | 160 | 140 |
| 5.0 | 13 | 16 | 13 | 140–160 | 140 |
| 5.5 | 12 | 15 | 14 | 140–160 | 140 |
| 6.0 | 12 | 15 | 11 | 140–160 | 140–160 |
| 6.5 | 11 | 16 | 11 | 140–160 | 140–160 |
| 7.0 | 10 | 15 | 10 | 140–160 | 140–160 |
| 7.5 | 10 | 14 | 11 | 140–160 | 140–160 |
| 8.0 | 12 | 16 | 12 | 140 | ~140 |
| 8.5 | 15 | 20 | 15 | 120–140 | 150 |
| 9.0 | 24 | 30 | 20 | 120(?) | |
| 9.5 | 30 | | | | |

bands of M-N64 and O-N64 intermediates after reduction of nitrotyrosine to aminotyrosine, we used the differences in the flash-induced transient absorbance changes at 360 and 430 nm in the two membrane preparations to detect a change in the protonation state of nitrotyrosine-64. Samples of bR-N64 and bR-N64R at pH 6.5 with the same absorbance at 530 nm were prepared, and the flash-induced transient absorbance changes from 340 to 450 nm were recorded in 5-nm steps. For perfectly matched samples, flash-induced absorbance depletion at 540 nm was identical; only samples differing less than 10% in 540-nm absorbance were used, and for these, we corrected the other absorbance changes accordingly. For each wavelength we subtracted the absorbance change of bR-N64R from the absorbance change of bR-N64 and plotted the difference as a function of wavelength (Figure 8). The resulting difference spectra closely resemble the difference spectra obtained by pH titration of nitrotyrosine (Scherrer & Stoeckenius, 1984) with an absorbance increase at 425 nm and a depletion at 360 nm and an isosbestic point around 380 nm. The 360-nm depletion and the increase at 425 nm are simultaneous. The half-time for the 425-nm absorbance increase is approximately 30 $\mu$s and for its decay around 10–15 ms. From the depletion at 540 nm and the absorbance change at 425 nm we calculate that 0.81 ± 0.15 mol of tyrosine-64 per mole of bR cycling

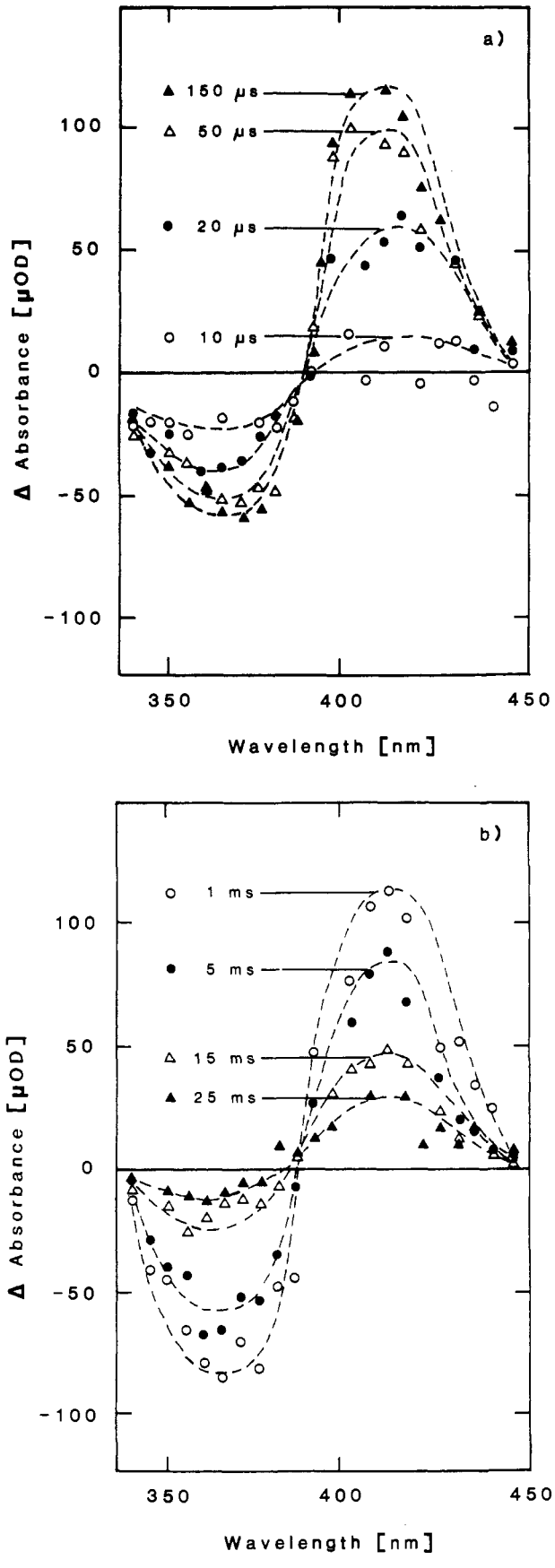


FIGURE 8: The flash-induced absorbance change at 20 °C in bR-N64 reduced was subtracted from the induced absorbance change in bR-N64 at (a) 10, 20, 50, and 150 μs and (b) 1, 5, 15, and 25 ms after the flash and plotted as a function of wavelength. bR-N64 and bR-N64R were resuspended in 10 mM NaCl, and the pH was adjusted to 6.5.

Table V: Light-Induced Proton Translocation Activity of Purple Membrane Preparations Incorporated into Vesicles of Asolectin[a]

| | rate [mol of $H^+$ (mol of bR)$^{-1}$ $s^{-1}$] | $H^+$/photon absorbed |
|---|---|---|
| bR | 0.5 | 0.25 |
| bR-N26 | 0.41 | 0.2 |
| bR-N26R | 0.35 | 0.18 |
| bR-N64 | 0.28 | 0.23 |
| bR-N64R | 0.18 | 0.14 |

[a] For details see Materials and Methods.

is transiently deprotonated during the photoreaction cycle.

*Kinetics of the M and O Intermediates in bR-N26 and bR-N26R Compared to bR.* The data are summarized in Tables I–IV. The rise kinetics of M-N26 and M-N26R are essentially the same as those of M and show the same pH dependence (Figure 3). The fast component in the M-N26 decay is roughly 2 times slower than in M, and this difference is somewhat reduced in M-N26R (Figure 4b). A decrease in the rate of the fast component is seen in M-N26 but not in M when the pH is decreased from 5.0 to 4.0. Again the difference is less in M-N26R. The slow-decay component of M-N26 is roughly 3–4 times slower and in M-N26R 2 times slower than that in M between pH 4.0 and pH 8.5. All three rates decrease sharply at higher pH with the same apparent p*K* (Figure 4b). The extent to which the slow component contributes to the total absorbance shows the same pH dependence for all three preparations (Figure 5b).

The kinetics of the O intermediate are little affected by nitration of Tyr-26. Only the decay of O-N26 is slightly slower than that of O, and this effect is reversed by reduction. We detected no difference in the absorbance change caused by the M intermediate between bR-N26 and bR-N26R in the microsecond time range. This indicates that Tyr-26 does not change its protonation state during this time. The M kinetics in the millisecond range are not the same for bR-N26 and bR-N26R, and therefore, no information about the protonation state of Tyr-26 could be obtained in this time range.

*Effects of Nitration on the Activity of Proton Pumping.* Table V shows that all modified preparations, when incorporated into lipid vesicles, showed light-driven proton translocation. The apparent pumping rates are slower than those seen in control bR vesicles by maximally a factor of ~2.5. These differences cannot be accounted for by differences in the proton permeability of the vesicle, because the proton gradients in all preparations relax at approximately the same rate and the initial rates measured should not be strongly affected by the passive permeability in any case.

We have shown above that the reduced extent of spectroscopically observed light adaptation in bR-N64 is only apparent and should not affect the pump activity, nor should the increase in cycling time, because we are working at light intensities well below saturation. However, the reduced extinction coefficients of bR-N64 and bR-N64R must reduce the pump efficiency. This is corrected for in the calculated quantum yield (column 3 in Table V), and the modified preparations still show a somewhat lower pumping activity.

Nevertheless, we cannot attribute the lower rates to an intrinsic lower activity of the modified preparations because we cannot rule out that they are caused by differences in the geometry of the vesicle, e.g., a larger number of multilamellar vesicles and/or a difference in the proportion of bR molecules oriented right side out and inside out compared to the control. A considerable amount of misoriented bR and/or multilamellar vesicles apparently is present also in the control preparation, because the calculated quantum efficiency for proton

translocation into the vesicles is less than half of what would be expected for perfectly oriented, unilamellar vesicles (Bogomolni et al., 1980; Govindjee et al., 1980). The relative efficiency of proton pumping by the modified preparations can only be answered by careful further studies; preliminary results with membranes absorbed to black lipid films, obtained in collaboration with E. Bamberg, have not shown significant differences from unmodified purple membrane preparations.

## Discussion

Protonation changes of tyrosine residues in the microsecond and millisecond time range are thought to play a controlling role in the kinetics and/or stoichiometry of the photoreaction and proton-pumping cycle of bR. This conclusion is based partly on the apparent p*Ks* of photocycle kinetic components and partly on transient UV-absorption changes indicating tyrosine deprotonation (Hess & Kuschmitz, 1979; Kalisky et al., 1981; Rosenbach et al., 1982; Fukumoto et al., 1984; Hanamoto et al., 1984; Bogomolni et al., 1978; Rafferty, 1979; Bogomolni, 1980; Kuschmitz & Hess, 1982). It has led several groups to investigate the effects of tyrosine modifications on these parameters (Konishi & Packer, 1978; Campos-Cavieres et al., 1979; Scherrer et al., 1981; Lam et al., 1983; Lemke & Oesterhelt, 1981; Rosenbach et al., 1982; Lemke et al., 1982). Combining observations of others with their own data, Kalisky et al. have postulated that deprotonation of a tyrosine residue is a prerequisite for deprotonation of the Schiff base in the L → M transition (Kalisky et al., 1981). Hanamoto et al., however, suggest that this conclusion is probably incorrect, because, at least under their conditions, M formation is faster than tyrosinate formation (Hanamoto et al., 1984). Instead, they suggest that a group with a p*K* of 9.6, which may or may not be a tyrosine, allows a faster decay of L to M when it is unprotonated than when it is protonated. Others have postulated that a tyrosine acts as the donor group for reprotonation of the Schiff base (Konishi & Packer, 1978).

Our own results in general agree with the earlier observations. They are, however, more extensive and allow additional conclusions because the modifications are restricted to a specific residue in each preparation. Together with the results of our earlier paper, they may be briefly summarized as follows. At pH 6.0 under illumination only Tyr-64 is nitrated, and this causes a >30-nm blue shift of the bR chromophore and shifts the p*K* of the Schiff base from pH >12 to pH <10. The M rise becomes slightly faster with no change in pH dependence, while the decay is slower. The apparent p*K* for its pH dependence shifts from 9.0 for M to 7.5 for M-N64. The O-N64 rise is 2–3 times slower than that of O, and its decay is 10 times slower. Nitrated Tyr-64 deprotonates in the L → M transition, and its reprotonation is slower than that of the O rise but slightly faster than the slow M decay. None of these changes is affected by subsequent reduction.

At pH 9.0 in the dark only Tyr-26 is nitrated, and this shifts its p*K* and that of the Schiff base from approximately 12.0 to 10.6, and both shifts are reversed by subsequent reduction. The only effect on the photocycle is a reduction in the decay rate of the M intermediates, which is only partially reversed by reduction. We conclude that in the dark Tyr-26 may have a slightly lower p*K* than the other tyrosines (but still >11.0) and that its deprotonation may lower the p*K* of the Schiff base. However, its p*K* change by about 1.5 units does not affect the pH dependence of the photocycle kinetics significantly and, Tyr-26 apparently does not deprotonate in the microsecond time range.

Tyr-64 transiently deprotonates during the L → M transitions at least in bR-N64, and the observation that it is selectively nitrated at pH 6.0 in the light argues strongly that the same is true for bR since TNM is known to react only with the tyrosyl ion. Whereas Tyr-64 modification has pronounced effects on the chromophore absorption and photoreaction cycle kinetics, these do not depend on its p*K*. Therefore, these effects as well as the blue shift of the visible absorption maximum and the lowering of the Schiff base p*K* are presumably mediated by conformational changes in the protein, possibly caused by the addition of a bulky group to the phenol ring.

Neither Tyr-26 nor Tyr-64 can, therefore, be the postulated groups that control the M rise, nor can either be a proton donor group controlling its decay. Furthermore, since most earlier observations agree that only one tyrosine deprotonates during the late photocycle reactions and since our results show that this tyrosine is Tyr-64, we must conclude that none of the other Tyr residues is likely to be involved either. It is, of course, possible that simultaneous but opposite protonation changes of two tyrosines or the extremely rapid reversal of a protonation change could have escaped detection.

Several other groups have used tetranitromethane to modify Tyr residues in bR, but only Lemke et al. have identified the modified residues in the amino acid sequence (Lemke & Oesterhelt, 1981; Lemke et al., 1982). From their and our earlier work, one may, however, assume that under mild conditions, in addition to Tyr-26 and -64, only Tyr-131 and -133 are nitrated. If we compare the results under this assumption, the reported spectroscopic properties agree reasonably well with one exception. Rosenbach et al., using the Lemke–Oesterhelt preparation, report a much faster M formation in their nitrated bR, which is not pH-sensitive (Rosenbach et al., 1982). The reason for the difference to our observations is obscure, but the preparations may not be entirely comparable. Their conclusion that Tyr-26 controls the M rise is contradicted by our observations as already discussed. However, since their preparations also contained modified Tyr-64, we would expect that their preparations also underwent a similar or identical conformational change and their other results are not inconsistent with our interpretation.

Apparently, discrepancies exist between our results and the reported effects of tyrosine modification on the proton-pumping activity of bR. Lemke et al. have reported that modification of either Tyr-26 or Tyr-64 can inactivate the proton pump (Lemke et al., 1982). Their nitrotyrosine-26 preparation, however, also contained aminotyrosine in the 64, 131, and 133 positions, and while it is therefore not strictly comparable to our bR-N26 preparation, nevertheless, our results preclude the conclusion that nitration of Tyr-26 alone can inhibit pumping. They also report inactivation of the pump after conversion of the Tyr-64 residue to the mono-*p*-azobenzenesulfonic acid derivative of tyrosine, which disappeared after reduction to aminotyrosine. This is consistent with our observation that bR-N64R pumps protons. The reason for the reported inactivation by sulfonylation remains obscure and needs further investigation.

We have shown above that the spectroscopically apparent reduction in light adaptation of bR-N64 can be explained by a reduced effect of Tyr-64 nitration on the 13-cis isomer of retinal and that virtually complete conversion of the 13-cis to the all-trans isomer takes place upon illumination of dark-adapted bR-N64. However, the observation that the retinal extraction is strongly inhibited in bR-N64 remains unexplained. It is not obvious why nitration of Tyr-64 should cause this effect, and the possibility of additional modifications not observed by our analysis of spectroscopic changes and peptide fractionation must be considered. The probability that TNM

causes dimerization of tyrosine residues increases with decreasing pH (Lundblad & Noyes, 1984), and selective nitration of Tyr-64 is carried out at much lower pH than all other nitrations. If the dimerization does indeed occur, it can only involve Tyr residues on the same CNBr peptide because the peptide fractionation pattern is undisturbed. This suggests Tyr-79 and Tyr-83 as the most likely candidates, because they are separated by three residues and presumably located on the same $\alpha$-helix. We do not think it is likely that this dimerization, if it exists, is also responsible for the effects on the chromophore because these effects are also observed in the Lemke–Oesterhelt preparations containing nitrated Tyr-64 and these were obtained at alkaline pH. Nevertheless, the reduced extractability of retinal in bR-N64 needs further investigation.

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