PHOTOCHEMISTRY OF MONOMETHYLATED AND PERMETHYLATED BACTERIORHODOPSIN

RAJNI GOVINDJEE,* ZSOLT DANCSHAZY,* THOMAS G. EBREY,* COLIN LONGSTAFF,‡ AND ROBERT R. RANDO‡

*Departments of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801; and ‡Departments of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Methylation of the nonactive site lysines of bacteriorhodopsin to form permethylated bacteriorhodopsin does not interfere with the formation of the short wavelength intermediate M412 or light-induced proton release/uptake. The absorption spectrum is similar to that of the native bacteriorhodopsin. However, additional monomethylation of the active site lysine of bacteriorhodopsin causes a red shift of the absorption maximum from 568 nm in light-adapted bacteriorhodopsin [BR] to 630 nm. The photochemistry of active-site methylated BR does not proceed beyond the L-photointermediate. In particular, the photointermediate corresponding to M412 does not form, and there is no proton pumping. Moreover, there is no tyrosine deprotonation. Thus, the formation of an M-type photointermediate is required for proton pumping by BR.

INTRODUCTION

Bacteriorhodopsin (BR) is the chromoprotein responsible for light-activated proton pumping in *Halobacterium halobium*. It consists of a single polypeptide of 248 amino acid residues attached to a retinal moiety at lysine 216 via a Schiff base linkage, which is protonated (Khorana et al., 1979). Bacteriorhodopsin exists in two states: a dark-adapted state with λ_{max} 558 nm, and a light-adapted state with λ_{max} 568 nm. Upon light absorption the light-adapted form undergoes a photocycle during which the BR absorption at 568 nm decreases transiently and a series of spectroscopically distinct intermediates (*K*, *L*, *M*, *R*, and *O*) are formed. *K* is red-shifted with respect to the parent pigment and quickly decays (~1 μs) to a species slightly blue-shifted with respect to bacteriorhodopsin, termed *L*. *L* decays to a short wavelength, possibly heterogeneous species *M*, which then decays back to BR (Lozier et al., 1975; Stoeckenius et al., 1979; Xie et al., 1987). Two long wavelength long-lived species, *R* (Dancshazy et al., 1986) and *O*, can be seen under appropriate sample conditions. During the BR photocycle the Schiff base is deprotonated in forming the *M* state (Lewis et al., 1974; Aton et al., 1977) and proton release takes place into the medium at approximately this point (Lozier et al., 1976; Ort and Parson, 1979). The photocycle proceeds with a reprotonation of the Schiff base and proton re-uptake from inside the cell. Light-induced deprotonation of a tyrosine residue has also been shown to occur during the BR photocycle (Hess and Kuschmitz, 1979; Kalisky et al., 1981; Fukumoto et al., 1984).

Abercrombie and Khorana (1986) showed that acetylation of the ε-amino groups of Lys-30, -40, and -41 did not affect proton translocation in BR. Longstaff and Rando (1987) have shown that BR in which only the non active-site lysines are methylated (Permethylated bacteriorhodopsin; PMBR) shows normal proton pumping, and its absorption spectrum is similar to native BR. However, additional methylation of the active-site lysine results in a bathochromic shift of the absorption maximum to 630 nm. The active-site methylated bacteriorhodopsin (AMBR), when incorporated into phospholipid vesicles, does not show light-induced pH changes. Since the deprotonation/reprotonation of the Schiff-base is a key to the photocycling and probably proton pumping of bacteriorhodopsin, it was of interest to see the effect of methylation of the active site lysine on the BR photocycle and proton release/uptake. Our flash photolysis experiments show that both AMBR and PMBR undergo photocycling. Like BR, the photocycle of PMBR has an *M* photointermediate in the submillisecond time scale, and shows light-induced proton release and uptake. However, the photocycle of AMBR has no *M* type intermediate and does not show light-induced proton release/uptake, as measured with a pH sensitive dye. Also, while tyrosine deprotonation can be observed in BR and PMBR, it is not seen in AMBR.

MATERIALS AND METHODS

Purple membrane was prepared from *Halobacterium halobium* strain S9 according to the method of Becher and Cassim (1975). AMBR and

Dr. Dancshazy's permanent address is Institute of Biophysics, Biological Research Center, Szeged, H-6701, Hungary.

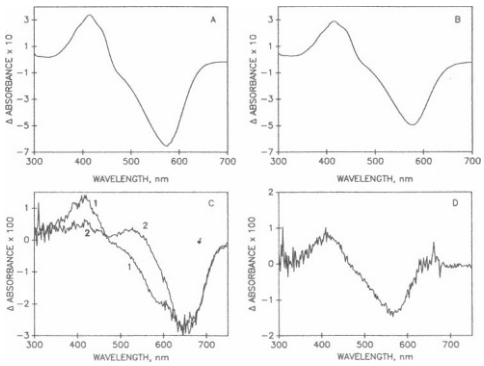

FIGURE 1 Photoflash-induced difference spectra measured with a Hewlett-Packard diode array spectrophotometer. (*A*) Bacteriorhodopsin: actinic light photoflash, λ's >520 nm; (*B*) permethylated bacteriorhodopsin; (*C*) active-site methylated bacteriorhodopsin: curve 1 (actinic light photoflash, λ's >520 nm) and curve 2 (photoflash, λ's >600 nm); and (*D*) difference between curves 1 and 2 in *C*. All samples in 150 mM KCl plus 50% glycerol; pH 7; −16°C.

PMBR were prepared according to the method of Longstaff and Rando (1987).

Flash-induced difference absorption spectra were measured either with a kinetic spectrophotometer or a Hewlett-Packard diode array spectrophotometer (model 8452A; Hewlett-Packard Co., Palo Alto, CA) having a spectral resolution of 2 nm. The actinic light was either provided from a Nd-Yag laser (10 ns, 532 nm, Quanta Ray DCR-11; Spectra Physics, Mountain View, CA) or a photoflash (150 μs half-pulse width) plus Corning long-pass filters (Corning Glass Works, Corning, NY) CS3-67 (λ's >520 nm), CS2-61 (λ's >600 nm), or CS2-58 (λ's >620 nm). Proton release and uptake were observed by measuring the absorbance changes of bacteriorhodopsin at 458 nm in the absence and presence of a pH sensitive dye pyranine followed by computer substraction of the two traces to obtain the absorbance change of the dye alone.

RESULTS

Bacteriorhodopsin

The photoflash-induced (actinic λ's >520 nm) difference absorbance spectrum of BR at −16°C was measured 1 s after a flash with a HP diode array spectrophotometer. The spectrum shows a ΔAmin centered at 570 nm and a ΔAmax at 410 nm (Fig. 1 *A*).

Permethylated Bacteriorhodopsin

The absorption properties of PMBR are similar to those of native BR (Longstaff and Rando, 1987). The absorption maximum is at 566 nm for the light-adapted form. The flash-induced difference spectrum of PMBR measured with the HP diode array spectrophotometer with a photoflash (actinic λ's >520 nm) shows a ΔAmax ~412 nm and a ΔAmin ~570 nm (Fig. 1 *B*).

Active-Site Methylated Bacteriorhodopsin

The absorbance maximum of AMBR is at ~630 nm (Longstaff and Rando, 1987). However, our sample probably contains some unmethylated BR (λ_{max} = 568 nm). Using a 532-nm laser excitation the flash-induced difference spectrum at 20°C measured ~1 ms after the flash

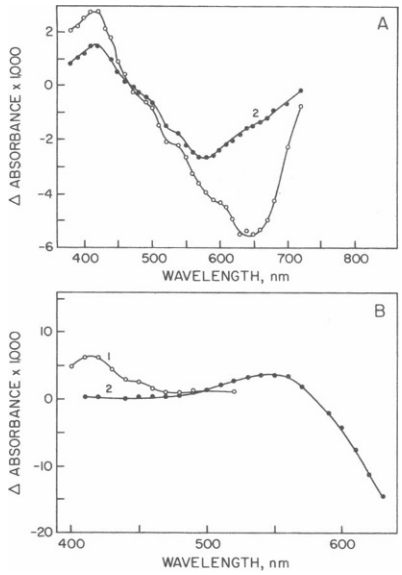

FIGURE 2 Difference absorption spectra of active-site methylated bacteriorhodopsin measured with a kinetic spectrophotometer. (*A*) Laser-induced (532 nm) difference spectra 1 ms (*curve 1*) and 40 ms (*curve 2*) after the flash. (*B*) Photoflash-induced difference spectra: actinic light λ's >520 nm (*curve 1*), and λ's >620 nm, (*curve 2*). 50 mM KCl, temperature 20°C, pH 7.

shows a ΔAmax ~410 nm and a minimum ~640 nm with a prominent shoulder ~570 nm (Fig. 2 *A*, *curve 1*). However, the difference spectrum measured after 40 ms shows a minimum at 570 nm and a ΔAmax at 410 nm, (Fig. 2 *A*, *curve 2*). The difference spectra measured with the HP diode array spectrophotometer have different features depending upon the cut-off filter used with the actinic flash. If CS 3-67 (λ's >520 nm) is used then the difference spectrum (Fig. 1 *C*, *curve 1*) looks basically the same as in Fig. 2 *A*, curve 1. However, if CS 2-61 (λ's >600 nm) is used then the difference spectrum has a ΔAmin ~640 nm, a ΔAmax ~550 nm, and a very small absorbance increase ~410 nm, (Fig. 1 *C*, *curve 2*). The difference spectra measured with the kinetic spectrophotometer together with the photoflash and one of two glass filters, CS 3-67 or CS 2-58, are shown in Fig. 2 *B*. The longer pass filter, CS 2-58 (λ's >620 nm) shows only a ΔAmax ~550 nm and no or very small absorbance increase at 410 nm (Fig. 2 *B*, *curve 2*). The actual position of the bleaching minimum could not be measured because of interference from the flash but it is >620 nm. In contrast, the spectrum obtained using CS 3-67 shows a ΔAmax ~410 nm (Fig. 2 *B*, *curve 1*).

Proton Release and Uptake. Laser-induced ($\lambda_{actinic}$ = 532 nm) proton release and uptake were measured using the pH indicator dye, pyranine. Fig. 3 (*left-hand panel*) shows the absorbance change measured at 410 nm for native BR, PMBR, and AMBR. The right-hand panel shows the corresponding absorbance change of the indicator dye, pyranine, measured at 458 nm; the decrease in absorbance indicates proton release followed by proton re-uptake. As seen in Fig. 3 *B*, PMBR shows

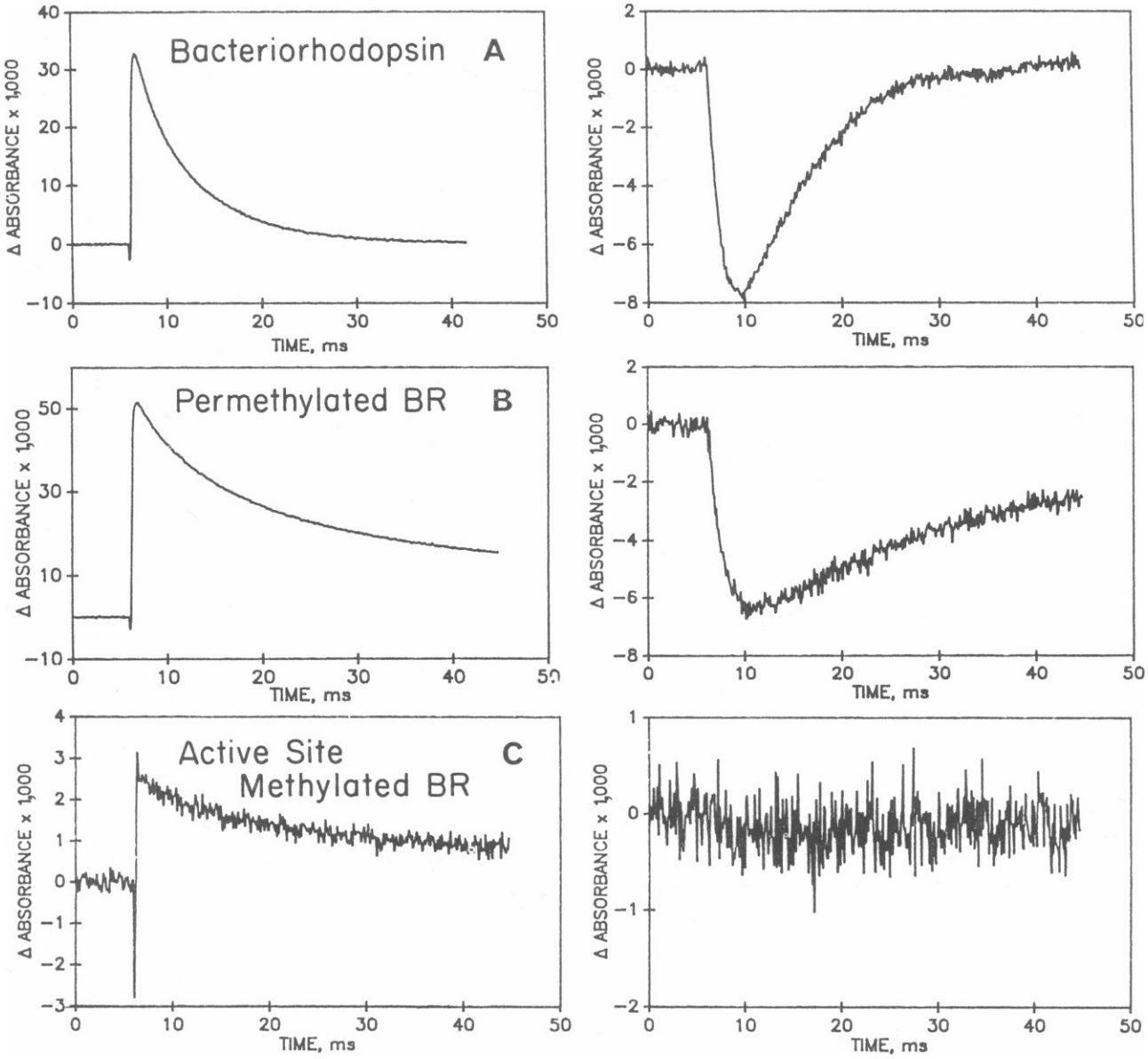

FIGURE 3 Laser-induced absorbance changes of bacteriorhodopsin (*A*), permethylated bacteriorhodopsin (*B*), and active-site methylated bacteriorhodopsin (*C*). Left-hand panel: ΔA 410 nm. Right-hand panel shows the corresponding absorbance changes of the pH-sensitive dye pyranine measured at 458 nm showing the proton release (decrease in absorbance) and reuptake; actinic light, λ actinic – 532 nm; 50 mM KCl, pH 7, 20°C.

normal flash-induced proton release and uptake; the proton uptake however is slower than native BR (Fig. 3 *A*). AMBR, on the other hand, shows no or very small proton changes (Fig. 3 *C*).

Tyrosine Deprotonation. Light-induced tyrosine deprotonation can be observed by measuring the absorbance changes at 296 nm. The absorbance change at 296 nm has a very rapid phase followed by a slow increase. The fast rise has been ascribed to chromophore isomerization, whereas, the slower phase to tyrosine deprotonation (Fukumoto et al., 1984). As seen in Fig. 4 *A* and *B* we could observe an absorbance increase due to the formation of a tyrosinate ion in BR and PMBR, but AMBR (Fig. 4 *C*) shows only a very small fast absorbance increase presumably due to chromophore isomerization, and no slow phase ascribable to tyrosine deprotonation.

DISCUSSION

The BR photocycle has been studied quite extensively and the different photointermediates characterized in terms of their absorbance maxima and lifetimes (Lozier et al., 1975, 1976; Kung et al., 1975; Stoeckenius and Bogomolni, 1982). These life-times range from nanosecond to several milliseconds. We cannot observe the early intermediates, *K* and *L*, with our kinetic spectrophotometer because their decay is very fast. However, we can observe the *M* type and subsequent photointermediates.

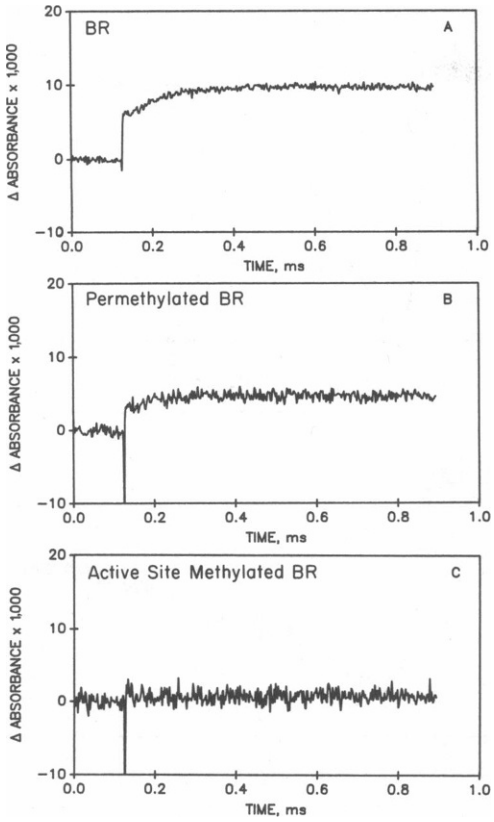

FIGURE 4 Laser-induced absorbance changes measured at 300 nm. (*A*) Bacteriorhodopsin; (*B*) permethylated bacteriorhodopsin; and (*C*) active-site methylated bacteriorhodopsin. Actinic λ = 532 nm; 50 mM KCl; pH 6.8; 25°C.

The isomerization of the chromophore and the deprotonation/reprotonation of the Schiff-base are key events in the photocycle and proton pumping by BR. Modification of the Schiff-base should give us an insight into these processes. Longstaff and Rando (1987) have shown that the methylation of the nonactive site lysines of BR to make permethylated BR does not affect its absorption spectrum. Flash photolysis of the PMBR shows that a short wavelength *M* intermediate is formed with ΔAmax ~410 nm and a bleaching minimum is present at 570 nm (Fig. 1 *B*), as for native BR (Fig. 1 *A*). However, additional monomethylation of the active site lysine causes a red shift in the absorption spectrum. Upon flash photolysis AMBR also undergoes a photocycle. The laser-induced difference spectrum in the millisecond time scale shows a ΔAmax ~410 nm and an absorbance minimum ~640 nm with a shoulder at ~570 nm; 40 ms later the absorbance minimum shifts to 570 nm and the maximum still remains at 410 nm although it is reduced in size (Fig. 2 *A*). Since the absorption maximum of methylated BR is red shifted by ~50 nm compared with BR, its photointermediates would also be expected to have absorbance maxima shifted to longer wavelengths relative to the corresponding BR photointermediates. L-AMBR has a ΔAmax at ~540–550 nm (see below), ~50 nm red-shifted compared with *L* which has a ΔAmax ~490–500 nm (Kalisky et al., 1981). Thus, it is unlikely that the ΔAmax at 412 nm represents M-AMBR.

Since the 532 nm laser flash is absorbed by both methylated and unmethylated species, we interpret the difference spectrum in Fig. 2 *A*, curve 1 as being a sum of 2 difference spectra. One spectrum comes from the photochemistry of the methylated BR with a bleaching minimum at 640 nm, and no short wavelength intermediate. The second spectrum is due to the photochemistry of a small amount of residual active site unmethylated BR with a bleaching minimum at 570 nm and a ΔAmax at 410 nm due to the *M* intermediate. After 40 ms the difference spectrum has a ΔAmax at 410 nm and a ΔAmin centered at 570 nm, (Fig. 2 *A*, *curve 2*), indicating that the recovery of the AMBR is faster than the unmethylated BR.

The difference spectra observed with the diode array spectrophotometer substantiate this interpretation. Since the absorbance maximum of AMBR is at 630 nm and that of native BR is at 570 nm, it was possible to provide an actinic flash using a photoflash plus long-pass filters to preferentially excite one or both the pigments depending upon the cut-off wavelength of the filter used. As seen in Fig. 1 *C*, curve 1, the difference spectrum with photoflash plus CS 3-67 is similar to the difference spectrum in Fig. 2 *A*, curve 1 (obtained with the laser flash) with a minimum at 640 nm, a shoulder at 570 nm, and a ΔAmax at 410 nm. However, when an actinic flash was provided with photoflash plus CS 2-61 the difference spectrum is as shown in Fig. 1 *C*, curve 2, with a minimum at 640 nm, and small absorbance increases at 530 and 410 nm. The difference between curves 1 and 2 in Fig. 1 *C* gives the difference absorption spectrum of BR (Fig. 1 *D*). Since the CS 3-67 filter transmits all the wavelengths longer than 520 nm it excites both pigments but the CS 2-61 filter which transmits beyond 600 nm preferentially excites the AMBR. Thus, when photoexcited, AMBR has a bleaching minimum at 640 nm and a ΔAmax at 530 nm in its difference spectrum measured 1 s after the flash at −18°C.

The 530 nm absorbance increase probably represents the formation of the photointermediate L-AMBR analogous to the *L* photointermediate of BR. Normally *L* cannot be detected under our conditions because the decay of *L* to *M* is very fast but, for AMBR, no deprotonation of the Schiff-base can take place and L-AMBR must decay back to the parent pigment without going through the deprotonated photointermediate M-AMBR; under these conditions, the decay of L-AMBR must be slowed down and thus we can observe the absorbance change due to L-AMBR on the millisecond time scale. The small absorbance increase observed at 410 nm (Fig. 1 *C*, *curve 2*) is caused possibly by the actinic effect of the white measuring beam of the diode array spectrophotometer causing unmethylated BR

to cycle. It is not seen in Fig. 2 *B* where the photoflash plus long-pass filters were used in conjunction with the kinetic spectrophotometer (where the measuring beam is monochromatic and of much weaker intensity). Fig. 2 *B*, curve 1 shows the presence of M412 when the CS 3-67 filter was used, but no M412 could be detected when CS 2-58 filter was used, (Fig. 2 *B*, curve *2*). Instead only the L-AMBR at 550 nm is seen. In fact, at 570 nm (the bleaching minimum for native BR) there is still an absorbance increase. The ΔAmax of L-AMBR is at 530 nm in Fig. 1 *B* whereas, it is ~550 nm in Fig. 2 *B*. This difference can also be ascribed to the actinic effect of the measuring beam. A small bleaching signal at 570 nm produced by the white measuring beam in the diode array spectrophotometer could cause a blue shift of the ΔAmax of L-AMBR from 550 to 530 nm.

As discussed above AMBR does not form an M intermediate. Since the proton release has been linked with *M* formation, (Lozier et al., 1975; Ort and Parson, 1979; Govindjee et al., 1980; Li et al., 1984; Krupinski and Hammes, 1985) it was of interest to see if AMBR shows light-induced proton release and uptake, and also to check the role of tyrosine(s) in the proton pump. Tyrosine deprotonation has been shown to occur during the BR photocyle (Bogomolni et al., 1978; Hess and Kuschwitz, 1979; Kalisky et al., 1981; Hanamoto et al., 1984). As seen in Fig. 3 *B*, PMBR shows flash-induced proton release-uptake and tyrosine deprotonation (Fig. 4 *B*). AMBR shows no or very small proton release and uptake (Fig. 3 *C*), and no tyrosine deprotonation (Fig. 4 *C*). Consistent with these results are previous observations that PMBR, but not AMBR, can establish a proton gradient across a membrane (Longstaff and Rando, 1987).

Kalisky et al. (1981) had suggested that tyrosine residues play an important role in the BR proton pump. They proposed that at low temperature tyrosine deprotonation precedes the Schiff-base deprotonation. Fukumoto et al. (1984) confirmed the reversible deprotonation of tyrosine(s) residue(s) at physiological temperatures. However, it was shown that the tyrosine deprotonation either lags behind or occurs simultaneously with the formation of M412 (Hanamoto et al., 1984; Dupuis and El-Sayed, 1985). Since there is no proton release and tyrosine deprotonation in AMBR, a central role for tyrosine in the BR proton pump can be retained only if its deprotonation follows or is coincident with Schiff base deprotonation. From these results it is not possible to decide if tyrosine deprotonation is a part of the proton pump or if it is an unrelated side event resulting from conformational changes of the protein as a consequence of isomerization and deprotonation of the Schiff base. Mogi et al. (1987) have shown using site directed mutagenesis that changing one by one the tyrosine residues in BR to phenylalanine has no discernable effect on its proton pumping ability.

In conclusion we can say that the substitution of the Schiff base proton with a methyl group allows a photocycle to proceed up to the formation of an L-like intermediate. However, the deprotonation of the Schiff base is obligate for the formation of the *M* photointermediate and proton release by BR.

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