The M Intermediate of *Pharaonis* Phoborhodopsin Is Photoactive

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ABSTRACT The retinal protein phoborhodopsin (pR) (also called sensory rhodopsin II) is a specialized photoreceptor pigment used for negative phototaxis in halobacteria. Upon absorption of light, the pigment is transformed into a short-wavelength intermediate, M, that most likely is the signaling state (or its precursor) that triggers the motility response of the cell. The M intermediate thermally decays into the initial pigment, completing the cycle of transformations. In this study we attempted to determine whether M can be converted into the initial state by light. The M intermediate was trapped by the illumination of a water glycerol suspension of phoborhodopsin from *Natronobacterium pharaonis* called *pharaonis* phoborhodopsin (*p*pR) with yellow light (>450 nm) at −50°C. The M intermediate absorbing at 390 nm is stable in the dark at this temperature. We found, however, that M is converted into the initial (or spectrally similar) state with an absorption maximum at 501 nm upon illumination with 380-nm light at −60°C. The reversible transformations *p*pR ⇔ M are accompanied by the perturbation of tryptophan(s) and probably tyrosine(s) residues, as reflected by changes in the UV absorption band. Illumination at lower temperature (−160°C) reveals two intermediates in the photoconversion of M, which we termed M′ (or M'_{404}) and *p*pR′ (or $p\text{pR}'_{496}$). A third photoproduct, $p\text{pR}'_{504}$, is formed at −110°C during thermal transformations of M'_{404} and $p\text{pR}'_{496}$. The absorption spectrum of M'_{404} (maximum at 404 nm) consists of distinct vibronic bands at 362, 382, 404, and 420 nm that are different from the vibronic bands of M at 348, 368, 390, and 415 nm. $p\text{pR}'_{496}$ has an absorption band that is shifted to shorter wavelengths by 5 nm compared to the initial *p*pR, whereas $p\text{pR}'_{504}$ is redshifted by at least 3 nm. As in bacteriorhodopsin, photoexcitation of the M intermediate of *p*pR and, presumably, photoisomerization of the chromophore during the M → M′ transition result in a dramatic increase in the proton affinity of the Schiff base, followed by its reprotonation during the M′ → *p*pR′ transition. Because the latter reaction occurs at very low temperature, the proton is most likely taken from the counterion (Asp^{75}) rather than from the bulk. The phototransformation of M reveals a certain heterogeneity of the pigment, which probably reflects different populations of M or its photoproduct M′. Photoconversion of the M intermediate provides a possible pathway for photoreception in halobacteria and a useful tool for studying the mechanisms of signal transduction by phoborhodopsin (sensory rhodopsin II).

INTRODUCTION

Some species of *Archaea*, bacteria and unicellular flagellated algae, have developed a light-induced motile response, known as phototaxis, of reacting to changes in the light environment and migrating to optimum light conditions. In *Halobacteria*, the light-driven proton pump bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971) was shown to mediate motile responses in cells, presumably through light-induced changes of the transmembrane potential as a light is turned on and off (Yan et al., 1992; Bibikov et al., 1993). In addition to bacteriorhodopsin, *Halobacteria* developed three other specialized retinal proteins structurally related to bacteriorhodopsin (Oesterhelt, 1998): halorhodopsin, a light-driven chloride pump (Matsuno-Yagi and Mukohata, 1977; Lanyi, 1990); sensory rhodopsin I (sRI) (Bogomolni and Spudich, 1982); and phoborhodopsin (Takahashi et al., 1985b; Tomioka et al., 1986) also called sensory rhodopsin II (sRII) (Wolff et al., 1986; Marwan and Oesterhelt, 1987). The photophobic receptor of *Natronobacterium pharaonis* is closely related (Scharf et al., 1992) to the phoborhodopsin of *Halobacterium salinarum* (former *halobium*) and is called *pharaonis* phoborhodopsin (*p*pR) or *pharaonis* sensory rhodopsin II (*p*sRII). Signal transduction in these specialized photoreceptors occurs through the activation of transducer proteins. These proteins are activated after phototransformation of sensory rhodopsins into their active signaling state (see reviews in Spudich et al., 1995; Hoff et al., 1997). This type of signal transduction apparently enables a bacterium to achieve a higher sensitivity compared to phototaxis mediated by bR. Sensory rhodopsins undergo cyclic photochemical transformations (Spudich and Bogomolni, 1988; Miyazaki et al., 1992; Chizhov et al., 1998) that are similar in many aspects to those of bacteriorhodopsin, including the formation of intermediates analogous to

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Abbreviations: bR, bacteriorhodopsin; *p*pR, phoborhodopsin (sensory rhodopsin II, sRII) from *Natronobacterium pharaonis*; K and M, intermediates of the photocycle of *p*pR analogous to the intermediates of the bacteriorhodopsin photocycle; M′, the primary photoproduct(s) of the M intermediate of *p*pR; *p*pR′, secondary photoproduct(s) of M. The subscripts in the designations M_{390}, M'_{404}, $p\text{pR}'_{497}$, and $p\text{pR}'_{504}$ indicates a maximum in the absorption spectrum of the photoproduct at low temperature (−160°C). Note that the photoproduct M′ is different from the short wavelength subspecies of M absorbing at 350 nm, which was described earlier by Shimono et al. (1998) and is also designated as M′ or $p\text{pR}_{M'}$.

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the intermediates of bR, K, L, M, N, and O (in sRI the last two states have not been detected). In both pigments the signaling state is formed upon light-induced deprotonation of the Schiff base, leading to formation of the M intermediate, which absorbs in the deep violet/near-UV. The M intermediate slowly (in seconds) thermally converts into the initial state.

The functions of the two photoreceptor pigments are different; sRI is maximally sensitive to orange light (590 nm), which produces an attractant reaction. The attraction to light facilitates the accumulation of cells in the light, which provides the energy that can drive proton transport by bR and chloride transport by halorhodopsin. Photoexcitation of the M intermediate of sRI, which absorbs at 370 nm, results in a photophobic response (Spudich and Bogomolni, 1984).

The function of phoborhodopsin (sensory rhodopsin II) is primarily to detect and help to avoid exposure to potentially harmful short-wavelength light (maximum absorbance at 480–490 nm) (Takahashi et al., 1985b; Wolff et al., 1986; Marwan and Oesterhelt, 1987; Hoff et al., 1997). Absorption of a light quantum by phoborhodopsin causes reversal of the direction of swimming (photophobic reaction). It was suggested that the M intermediate (as well as O) might be a signaling state of the pigment (Yan et al., 1991). As in the case of bacteriorhodopsin, in *p*pR the formation of the M intermediate involves the all-*trans* to 13-*cis* photoisomerization of the chromophore (Imamoto et al., 1992a), which initiates proton transport from the Schiff base to a nearby aspartic acid, Asp^{75} (Engelhard et al., 1996). The decay of M is slowed compared to the bR photocycle, presumably because of the absence of an efficient proton donor analogous to Asp^{96} (Iwamoto et al., 1999a). The decay of M can be accelerated by the addition of azide, which is similar to that in the D96N mutant of bacteriorhodopsin (Takao et al., 1998). M decay and formation of the O intermediate are accompanied by proton uptake, whereas O decay correlates with proton release to the bulk in sRII and *p*pR (Sasaki and Spudich, 1999; Iwamoto et al., 1999b). Sasaki and Spudich observed that net proton release and uptake occurred at the same (extracellular) side of the membrane in *H. salinarum* envelop vesicles under their conditions, and therefore little or no transmembrane proton transport occurred. Iwamoto et al. (1999b) suggested, on the other hand, that in their conditions proton uptake might occur from the cytoplasmic surface and proton release to the extracellular surface of *p*pR.

The photocycle of phoborhodopsin has been studied both by low-temperature steady-state (Imamoto et al., 1991; Hirayama et al., 1992) and room-temperature kinetic spectroscopy (Imamoto et al., 1992b; Miyazaki et al., 1992; Chizhov et al., 1998). However, it has not been established whether the M intermediate of phoborhodopsin is photoactive, and if it is, what the pathway and physiological response of its phototransformation are. Investigation of this reaction will provide a useful approach to study the mechanism of the photophobic response and its connection with the intramolecular proton transfer reactions.

In bacteriorhodopsin, photoexcitation of the M intermediate causes fast reprotonation of the Schiff base (Litvin et al., 1975; Druckmann et al., 1992) from the counterion Asp^{85} (Balashov and Litvin, 1981a; Takei et al., 1992) and transformation of the pigment back to its initial state through a nonpumping pathway (Karvaly and Dancshazy, 1977; Ormos et al., 1978; Litvin et al., 1981; Ludmann et al., 1999). Phototransformation of M (reviewed by Balashov, 1995) involves the formation of two primary photoproducts, P421 and P433 (Litvin and Balashov, 1977; Balashov and Litvin, 1981b), called also M′ (Hurley et al., 1978), and several subsequent thermal intermediates, P565, P575, and P585 (or bR′), which are in turn photoactive (Balashov and Litvin, 1981a; Balashov et al., 1988).

In the case of sRI, the phototransformation of the M intermediate is utilized to provide a second transient signaling pigment (Spudich and Bogomolni, 1984; Hoff et al., 1997; Takahashi et al., 1985a): light absorption by the initial state (maximum at 587 nm) produces a positive motile response, whereas absorption of light by its photointermediate M (maximum at 373 nm) results in a negative (photophobic) response.

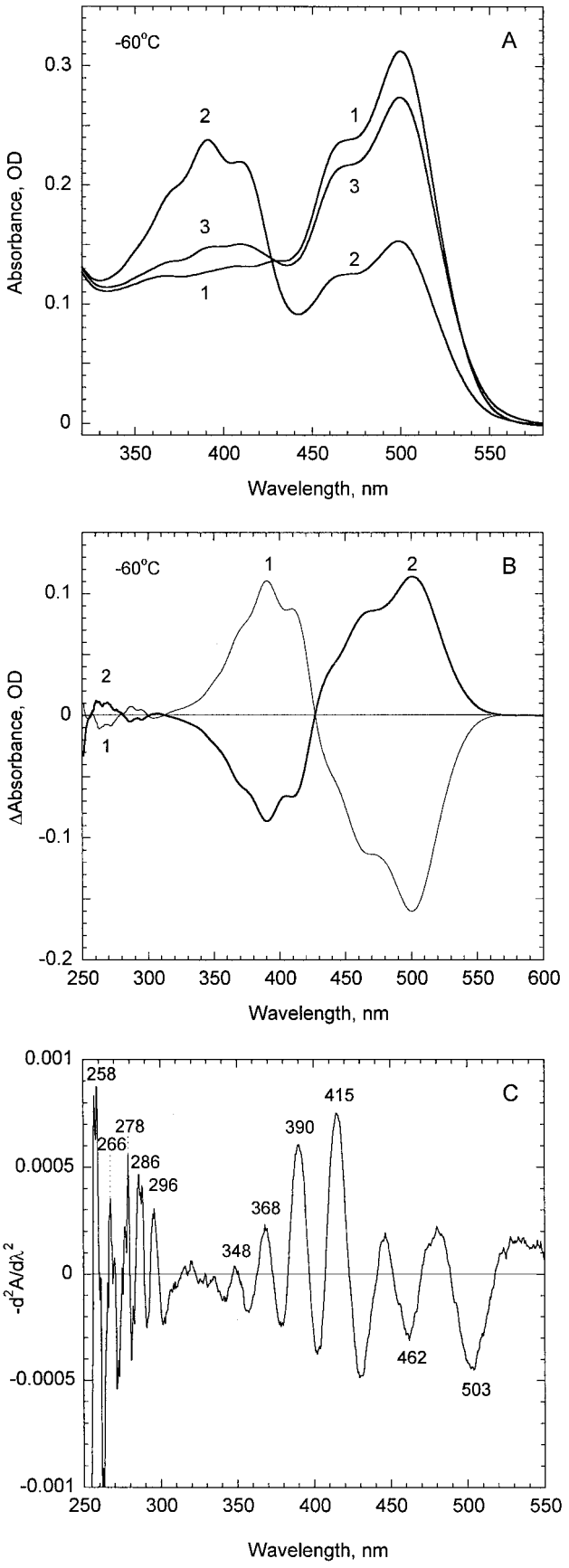
In this study we examined the photoactivity of the M intermediate of *p*pR at low temperatures, using *p*pR expressed in *Escherichia coli* and solubilized in a detergent (Shimono et al., 1997; Iwamoto et al., 1999b).

MATERIALS AND METHODS

Expression of *p*pR in *E. coli* was detected as described earlier (Shimono et al., 1997). The pigment was purified as described by Iwamoto et al. (1999). Stock suspension contained pigment in 0.5% *n*-dodecyl-β-D-maltoside (DM), 100 mM KCl, and 50 mM Tris buffer (pH 8.0). For low-temperature measurements it was diluted with two parts glycerol. The final pH was 7 (at 25°C). A homemade cryostat enabled us to perform measurements at temperatures from 25°C to −160°C in the 250–750-nm spectral range. The pathlength of the cuvette was 2 mm. Spectra were recorded on a Cary-Aviv 14DS UV-VIS spectrophotometer. Bandwidth of the monochromator was 1 nm. Spectra were recorded in 1-nm or 0.5-nm steps. The latter was mainly used to resolve the light-induced changes in the UV. In addition to the conventional methods of low-temperature absorption spectroscopy, we also used derivative spectroscopy. The second derivative ($-d^2A/d\lambda^2$) spectra significantly improved the resolution of overlapping vibrational bands (Balashov et al., 1991b). They are also helpful in the detection of small but narrow bands because the intensity of a band in the second derivative spectrum is inversely proportional to the square of the half-width of the absorption band. Application of the second derivative technique enabled us to easily determine the fraction of *p*pR in the photosteady-state mixture with its bathoproduct K and to resolve the vibrational structure of the absorption spectra of *p*pR and its photoproducts.

RESULTS

The M intermediate was formed upon illumination of *p*pR with 450–550-nm light at −50°C. About 50% of the pigment was transformed into M under these conditions (Fig. 1



A, *curves 1* and *2*). A larger amount of the pigment can be converted into M upon illumination of the sample at 450–550 nm while the temperature is decreased from −20°C to −50°C. However, a small fraction of other intermediates may be trapped along with M under these conditions, particularly an O-like species absorbing around 560 nm. Therefore we used illumination at −50°C to form M.

The absorption maximum of *p*pR is at 501 nm at −60°C, whereas M has its maximum at 390 nm in the M minus *p*pR difference spectrum (Fig. 1 *B*). The difference spectrum of M minus *p*pR at −60°C indicates that transformation of *p*pR into M is accompanied by an increase in the intensity of the 296-nm and 286-nm bands and a decrease of absorbance around 260–270 nm (Fig. 1 *B*, *curve 1*, and Fig. 2, *A* and *B*, *curve 1*). Interestingly, the changes in the UV accompanying formation of the M intermediate in *p*pR were different from those observed upon formation of the M intermediate in bacteriorhodopsin (Fig. 2 *B*, compare *curves 1* and *2*).

The vibrational structure of *p*pR and its M photoproduct can be resolved using the second derivative of the M minus *p*pR difference absorption spectrum (Fig. 1 *C*). Transformation of *p*pR into M was accompanied by a decrease in the 503-nm and 462-nm vibronic bands of *p*pR and the formation of 415-, 390-, 368-, and 348-nm vibronic bands of M (Fig. 1 *C*). In the UV region several peaks appeared in the second derivative spectrum at 296, 286, 278, 266, and 258 nm, which were caused by perturbation of aromatic residues in the *p*pR → M transition.

Photoconversion of M at −60°C and at −160°C: formation of photointermediates M′ and ppR′

The M intermediate is stable at −60°C in the dark (absorption spectrum does not change upon incubation for 30 min in the dark at −60°C). However, illumination of a sample containing M at −60°C with 360–420-nm light caused a transformation of most of M back to the pigment's initial state or a spectrally isochromic state (Fig. 1 *A*, *curve 3*), indicating that M is photoactive. The absorption maximum of the pigment photoregenerated from M at −60°C (Fig. 1 *B*, *curve 2*) is at 501 nm. It practically coincides with the maximum of the initial pigment. The absorption changes in

FIGURE 1 Absorption changes accompanying the formation and photoconversion of the M intermediate of *p*pR. (*A*) Absorption spectra measured at −60°C: (*1*) initial *p*pR; (*2*) after 5 min of illumination at 450–550 nm at −50°C to convert *p*pR into M and cooling to −60°C; 3, after 5 min of illumination at 360–420 nm at −60°C. (*B*) Difference spectra of formation (*1*) and photoconversion (*2*) of M. Spectra 1 and 2 were obtained, respectively, as the difference spectrum 2 minus 1 and spectrum 3 minus 2 of *A*. (*C*) Resolution of the vibronic bands of *p*pR and M at −60°C using the second derivative: $-d^2A/d\lambda^2$ of the difference spectrum of transformation of initial *p*pR into M at −60°C (of spectrum 1 in *B*).

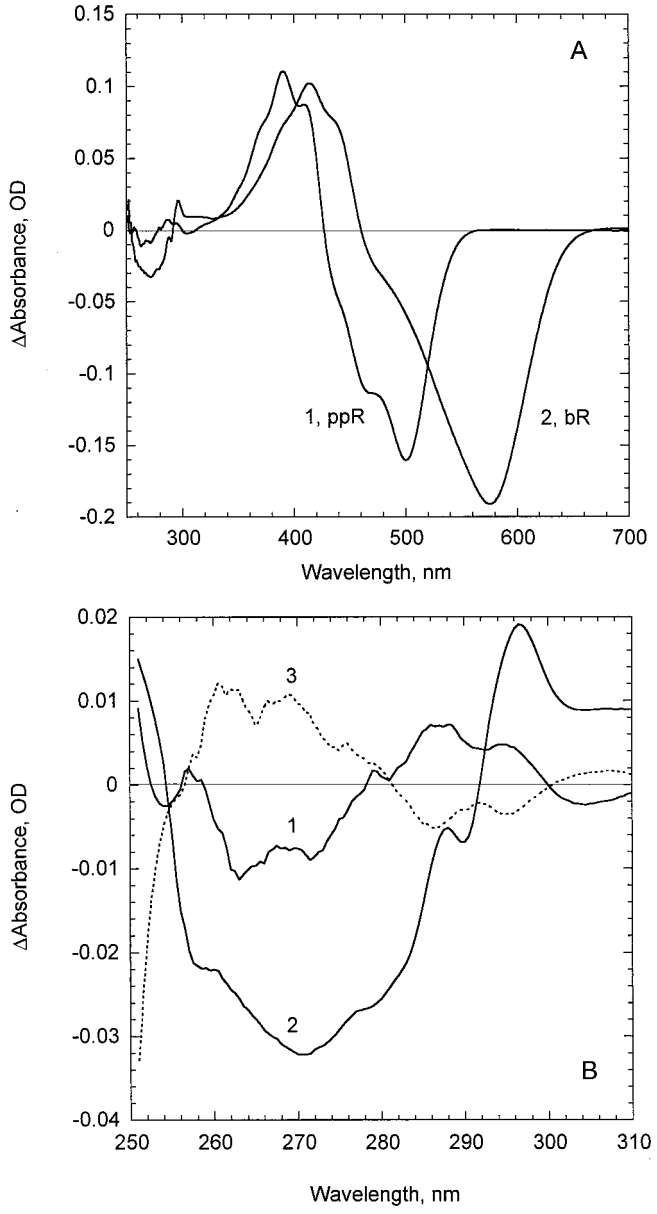


FIGURE 2 (*A*) Comparison of the absorption changes accompanying the formation of the M intermediate in *p*pR (*curve 1*) and bR (*curve 2*) at −50°C. Both samples contained 30 mM KCl and 15 mM Tris-buffer (pH 7.0). The difference spectrum of the bR to M transition in UV is similar to the earlier published spectrum (Sabés et al., 1984). (*B*) The UV region of the difference spectra accompanying (*1*) the formation of the M intermediate in *p*pR (Fig. 1 *B*, *curve 1*), (*2*) the formation of the M intermediate in bR at −50°C, and (*3*) photoconversion of the M intermediate in *p*pR (Fig. 1 *B*, *curve 2*).

the UV were reversed upon photoconversion of M back to *p*pR at −60°C (Fig. 2 *B*, *curve 3*).

Experiments at lower temperature (−160°C) revealed intermediates in the photoconversion of M. Illumination of the sample containing M at −160°C (Fig. 3 *A*, *curve 1*) resulted in a transformation of M into a long-wavelength species (Fig. 3 *A*, *curve 2*). This indicates that light-induced protonation of the Schiff base takes place, even at −160°C. The absorption maximum of the photoproduct in the difference spectrum (Fig. 3 *B*, *curve 1*) is at 496 nm; it is blueshifted 5 nm compared to the maximum of the initial *p*pR at −160°C (501 nm). We call this species $p\text{pR}'_{496}$.

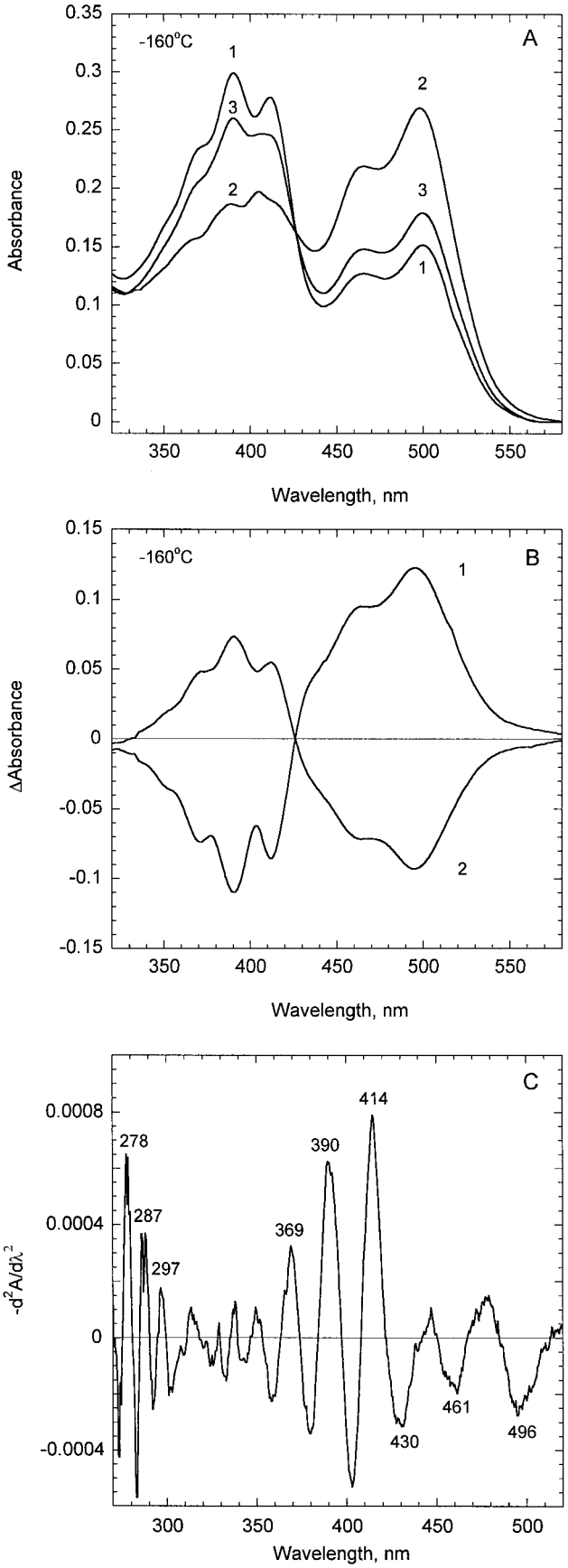
The photoproduct $p\text{pR}'_{496}$ differs from the initial *p*pR not only in its blueshifted spectrum, but also in its photochemical properties. Illumination of $p\text{pR}'_{496}$ at −160°C resulted in a conversion of the main fraction of $p\text{pR}'_{496}$ back to M (Fig. 3 *A*, *curve 3*). The second derivative of the difference spectrum clearly showed minimum at 496 nm and characteristic bands of M at 414, 390, and 369 nm (Fig. 3 *C*) due to the $p\text{pR}'_{496} \rightarrow$ M photoconversion.

The initial *p*pR at the same temperature (−160°C) undergoes phototransformation into its bathoproduct K (Fig. 4 *A*). From the amplitude of vibronic bands in the initial *p*pR and the photo-steady-state mixture of *p*pR and K produced by 460-nm light (Fig. 4 *B*), the fraction of K (52 ± 2%) and the ratio of the quantum yield of the forward and back reactions ($\phi_1/\phi_2 = 0.7 \pm 0.1$) can be estimated. The values obtained are similar to those for the primary light reaction in bacteriorhodopsin (Balashov et al., 1991b).

Besides $p\text{pR}'_{496}$, illumination of M at −160°C produced another photoproduct, which absorbed at shorter wavelengths. We term this photoproduct M′ (or M'_{404}). It is characterized by bands at 380 and 404 nm that appear in the absorption spectrum after illumination of M at −160°C (see Fig. 3 *A*, *curve 2*). These sharp bands are well resolved in the second derivative spectrum (see maxima at 380, 404, and 417 nm in Fig. 5 *A*, which are absent in the spectrum of M in Fig. 3 *C*). The difference absorption spectrum accompanying formation of M′ from M shows maxima at 402 and 378 nm due to the M′ formation (Fig. 5 *B*) and minima at 390, 368, and 412 nm, which are close to the position of vibronic bands of initial M. The photoproduct M'_{404} did not disappear after photoconversion of $p\text{pR}'_{496}$ back to M and thus is distinct from $p\text{pR}'_{496}$. Similar to M, M'_{404} absorbs at short wavelengths and hence has an unprotonated Schiff base. More details on the spectrum of M'_{404} were obtained upon thermal conversion of this intermediate (see below).

Thermal conversions of ppR'_{496} and M'_{404}

Increasing the temperature of the sample containing $p\text{pR}'_{496}$ and M'_{404}, from −160°C to −110°C, and cooling back to −160°C resulted in an increase of absorbance and a redshift of the absorption maximum of the photoproduct from 496 to 504 nm (Fig. 6 *A*, *curves 1* and *2*). A subsequent increase in the temperature to −60°C and cooling back to −160°C caused a blueshift of the absorption maximum from 504 to 501 nm (Fig. 6 *A*, *curve 3*). The latter is peculiar to the initial *p*pR at −160°C. These data indicate that during thermal conversions of M′ and $p\text{pR}'_{496}$ an intermediate is formed at −110°C, with the absorption maximum redshifted compared to initial *p*pR (to at least 504 nm). We will



call this photoproduct $p\mathrm{pR}'_{504}$. At −60°C all intermediates converted into the initial *p*pR (or to a spectroscopically identical species).

The difference absorption changes accompanying thermal conversions of M′ and $p\mathrm{pR}'_{496}$ with an increase in the temperature from −160°C to −60°C are shown in Fig. 6 *B*, curve 1. The minima at 362, 382, 404, and 420 nm correspond to the vibronic bands of M'_{404}. Two bands, at 382 nm and 404 nm, have almost the same amplitude. The positive band with the maximum at 509 nm is composed of two difference spectra caused by two thermal transitions: $\mathrm{M}'_{404} \rightarrow p\mathrm{pR}$ and $p\mathrm{pR}'_{496} \rightarrow p\mathrm{pR}$. The thermal transformation of M'_{404} can be observed separately from the transformation of $p\mathrm{pR}'_{496}$ (Fig. 6 *B*, *curve 2*) when most of $p\mathrm{pR}'_{496}$ is photoconverted back to M at −160°C, as shown in Fig. 3 *A*, curve 3. As the sample containing M'_{404} was warmed to −60°C, the M'_{404} intermediate underwent transformation into a species with an absorption maximum at 503 nm, which was probably mostly initial *p*pR. A small redshift from 501 to 503 nm in this spectrum was most likely caused by a residual amount of $p\mathrm{pR}'_{496}$ converting to *p*pR. As one can see from the difference spectra in Fig. 6 *B* (*curves 1* and *2*), the photoproduct M'_{404} has a distinctive vibrational structure with the bands at 417, 404, 381, and 365 nm, which overlap but are clearly different from the bands of M at 414, 390, and 369 nm (Fig. 6 *B*, *curve 3*). The two long-wavelength bands of M'_{404} are redshifted and narrower compared to the 414-nm and 390-nm bands of M.

DISCUSSION

Features of M photoconversion in ppR

The results presented above clearly indicate that the M intermediate of *pharaonis* phoborhodopsin (*pharaonis* sRII) is photoactive. The phototransformation of M involves formation of at least three intermediates, M'_{404}, $p\mathrm{pR}'_{496}$, and $p\mathrm{pR}'_{504}$. M'_{404} presumably is the photoproduct of the primary light reaction of M, which most likely involves isomerization of the chromophore. The sharper vibronic bands of M'_{404} indicate that the chromophore is more planar in M'_{404} than in M, consistent with the 13-*cis* → all-*trans* isomerization. The photoreaction M → M′ results in a dramatic increase in the proton affinity of the Schiff base because the long-wavelength species $p\mathrm{pR}'_{496}$ with a protonated Schiff base appears even at −160°C, whereas reprotonation of the Schiff base during thermal decay of M occurs only above

FIGURE 3 Photoconversion of the M intermediate of *p*pR at −160°C. (*A*) (*1*) Spectrum containing ~60% M and 40% *p*pR. (*2*) After 5 min of illumination at 360–420 nm at −160°C. (*3*) After 5 min of illumination at 450–550 nm. (*B*) Difference spectra of (*1*) photoconversion of M at −160°C (spectrum 2 − spectrum 1 in *A*); (*2*) photoconversion of *p*pR′ into M (spectrum 3 − spectrum 2). (*C*) Minus second derivative of the difference spectrum of photoconversion of *p*pR′ at −160°C ($-\mathrm{d}^2/\mathrm{d}\lambda^2$ of spectrum 2 in *B*).

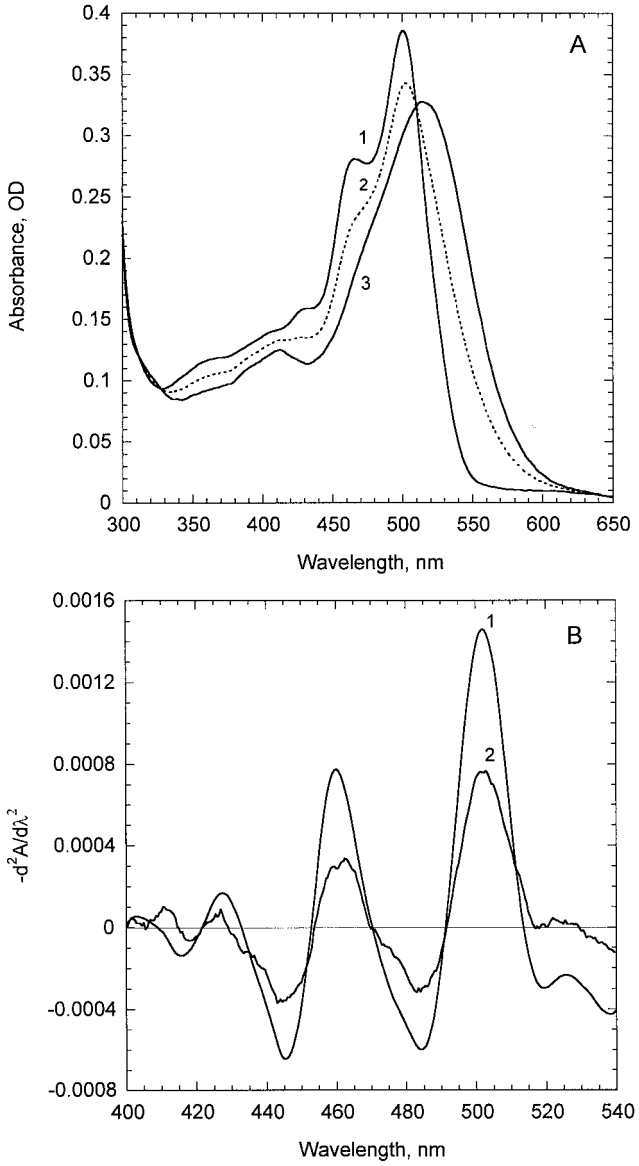


FIGURE 4 Phototransformation of the initial *p*pR at −160°C. (*A*) Absorption spectra (*1*) of *p*pR; (*2*) after illumination for 15 min at 460 nm; (*3*) of calculated spectrum of the bathoproduct K, assuming that 52% of *p*pR is transformed into K upon illumination with 460-nm light. (*B*) *Curves 1* and *2*, minus second derivatives of spectra 1 and 2 of *A*, respectively. From the decrease of the amplitudes of 503-nm and 460-nm vibronic bands of *p*pR after illumination, the fraction of the photoproduct K can be estimated as 0.52 ± 0.02. From the photo-steady-state concentrations of *p*pR and K and the calculated spectrum of K (*A, curve 3*), the quantum efficiency ratio of the forward and back light reaction, *p*pR ⇔ K, can be estimated (Balashov et al., 1991b) as 0.7 ± 0.1. This estimate is approximate because it does not take into account the heterogeneity of the bathoproduct K (Hirayama et al., 1992).

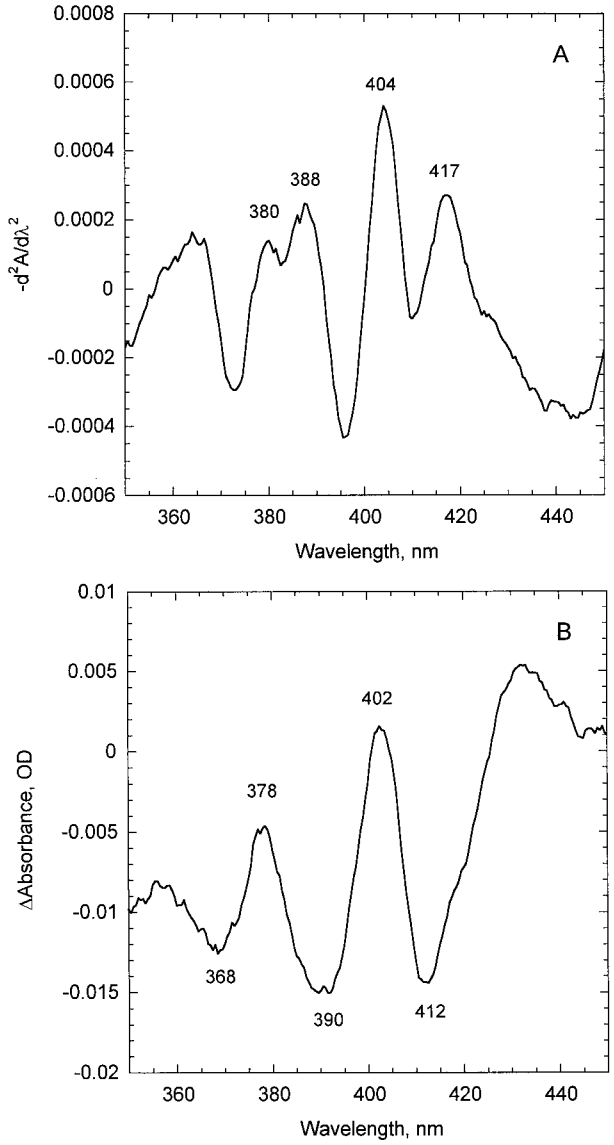


FIGURE 5 (*A*) Minus second derivative of the absorption spectrum of the sample containing M after its irradiation with 360–420-nm light at −160°C (of *spectrum 2* in Fig. 3 *A*). The 404-nm and 380-nm bands belong to the photoproduct M'_{404} formed upon 360–420-nm irradiation of M at −160°C (these bands are absent in the spectrum of M (Fig. 1 *C*). (*B*) Difference spectrum M'_{404} minus M. The spectrum was obtained as a result of subtraction of the absorption changes caused by the transformation of M into *p*pR′ (*spectrum 2* in Fig. 3 *B*, taken with a minus and multiplied by a scaling factor, 1.29) from the difference spectrum caused by the transformation of M into both *p*pR′ and M′ (*spectrum 1* in Fig. 3 *B*).

−40°C. In the M′ → *p*pR′ transition the proton is most likely taken from the counterion, Asp^{75}. The data indicate that phototransformation of M in *p*pR, like the phototransformation of M in bR (Litvin and Balashov, 1977; Balashov and Litvin, 1981b; Balashov, 1995), occurs through a pathway that is different from that used in the thermal transformation of M (Fig. 7). The simplest scheme of M photoconversion inferred from our data would be the following linear sequence:

$$M_{390} \xrightarrow{h\nu} M'_{404} \xrightarrow{-160^\circ C} ppR'_{496} \xrightarrow{-110^\circ C} ppR'_{504} \xrightarrow{-60^\circ C} ppR_{501}$$

However, we cannot exclude the possibility that the M intermediate and its primary photoproduct(s) M′ are heter-

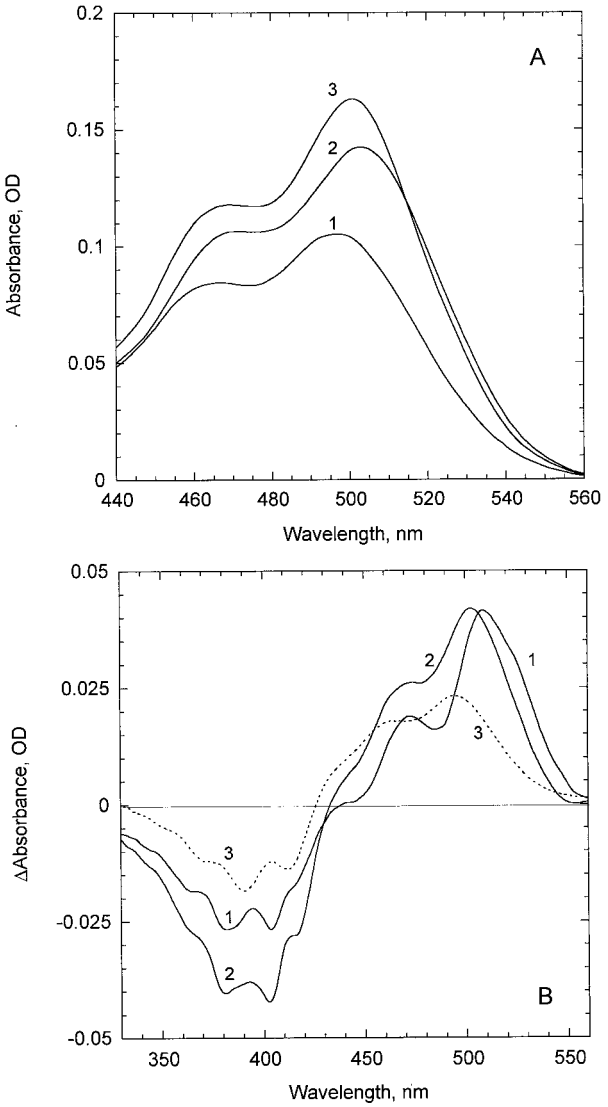


FIGURE 6 Thermal conversions of M'_{404} and ppR'_{496}. (*A*) Absorption spectra of the photoproducts of M produced (*1*) by illumination of the sample containing M at −160°C with 360–420-nm light (same as *spectrum 1* in Fig. 3 *B*); (*2*) after a subsequent increase in the temperature to −110°C and cooling back to −160°C; (*3*) after an increase in the temperature to −60°C and cooling back to −160°C. (*B*) Difference spectra caused by thermal transformations of M'_{404} and ppR'_{496}. (*1*) Absorption changes observed in the sample containing M'_{404} and ppR'_{496} (*sample 2* in Fig. 3 *A*) produced by increasing the temperature from −160°C to −60°C and cooling back to −160°C. (*2*) Same as 1, but most of ppR'_{496} was converted back to M (*sample 3* in Fig. 3 *A*), so the changes are caused mostly by the thermal conversions of M'_{404}. For the purpose of comparison of the spectrum of M'_{404} and initial M, the difference spectrum of M minus ppR'_{496} is given (*curve 3*). This is the same spectrum as in Fig. 3 *B*, curve 2, but multiplied by −0.25 to scale it with the other spectra.

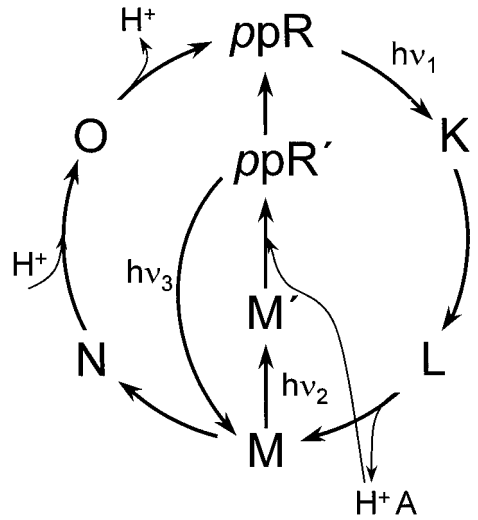


FIGURE 7 A simplified scheme of the photochemical conversions of *pharaonis* phoborhodopsin (*pp*R, or psRII). Excitation of the pigment with blue-green (500 nm light) causes a cyclic reaction in which deprotonation of the Schiff base and formation of the M intermediate take place (Hirayama et al., 1992; Chizhov et al., 1998). Presumably M and the subsequent O state act as signaling states for the photophobic reaction of the cell (Yan et al., 1991). Formation and decay of the O intermediate is accompanied by proton uptake and release, correspondingly (Sasaki and Spudich, 1999; Iwamoto et al., 1999b). Photoexcitation of M with a near-UV light (360–420 nm) results in the photoconversion of M back to the initial state. This pathway involves formation of several intermediates found in this study, the primary photoproduct of M, M'_{404}, and subsequent thermal products, ppR'_{496} and ppR'_{504}, designated as M′ and *pp*R′ in the scheme. Thermal transformation from M′ to *pp*R′ is accompanied by reprotonation of the Schiff base, presumably from its counterion, Asp^{75}, designated by letter A. The photoproduct *pp*R′ is photoactive. Upon absorption of a light quantum it is transformed into M.

ogeneous and that there are parallel pathways in the M photoconversion. Evidence for this is that at −160°C part of M is photoconverted into ppR'_{496}, whereas some fraction of the pigment stays in M'_{404}. This indicates the existence of two M′ intermediates with different thermal stabilities: one is transformed into ppR'_{496} at −160°C, and the other (M'_{404}) is stable at this temperature. To observe this less stable photoproduct, we have to examine the photoreaction of the M intermediate of *pp*R at lower temperatures. In bacteriorhodopsin, irradiation of M at −190°C does result in the formation of two primary photoproducts with different thermal stabilities (Balashov and Litvin, 1981b; Takei et al., 1992; Friedman et al., 1994).

Because photoconversion of M into ppR'_{496} occurs at −160°C, it is unlikely that the required proton for this transition is taken from the frozen bulk solution; the proton might be transported from the counterion (Asp^{75}), as in the case of bR (Balashov and Litvin, 1981b; Takei et al., 1992). The results suggest that photoconversion of M interrupts the photocycle of *pp*R and converts the pigment back to the initial state through a pathway that bypasses the N and O intermediates. Deprotonation of Asp^{75} in the O → *pp*R transition is the rate-limiting step in the photocycle of *pp*R. Photoexcitation of M presumably induces proton transport from Asp^{75} back to the Schiff base and subsequent reformation of the initial state. This hypothesis is in agreement with a recent study of Schmies et al. (2000), which showed

that under certain conditions blue light illumination of *pharaonis* sRII (*p*pR) decreased the photocurrent generated by background green light illumination. These results indicate photoactivity of the M intermediate and phototransformation of M through a pathway different from its thermal conversion.

Photoproduct $p\text{pR}'_{496}$ is photoactive, and at −160°C it is transformed into M upon illumination. This is similar to the phototransformation of the photoproduct P565 formed at −160°C upon irradiation of the M intermediate of bacteriorhodopsin (Litvin and Balashov, 1977; Balashov and Litvin, 1981a). The redshifted photoproduct $p\text{pR}'_{504}$ may be analogous to the redshifted species P585, which is formed in the photoconversion of the M intermediate of bR at −60°C (Balashov and Litvin, 1981a; Balashov et al., 1988). The different species with a protonated Schiff base that are formed in the course of phototransformation of M into the initial state most likely represent certain steps in reformation of the Schiff base-counterion environment peculiar to the initial pigment. In bR they differ in hydrogen bonding of the protonated Schiff base and its counterion with internal water molecules that stabilize the ion pair (Maeda et al., manuscript submitted for publication). These water molecules participate in the formation of the M intermediate of bacteriorhodopsin (Luecke et al., 1999a). Internal water molecules might be involved in the photoreactions of *p*pR and its M intermediate as well and might participate in the formation of the $p\text{pR}'_{496}$ and $p\text{pR}'_{504}$ species, along with conformational changes of other important groups involved in the formation of M.

Implications of photoreversibility of the M intermediate on signal transduction in phoborhodopsin

In bacteriorhodopsin phototransformation of M prevents transmembrane proton transport. Based on the similarity of the photoreactions of the M intermediate in *p*pM and bR, we suggest that photoconversion of M into *p*pR might result in deactivation of the photoreceptor's signaling state. There is evidence that phototransduction of a light-induced signal occurs through the direct interaction of pigment with its phototransducer protein HtrII (Sasaki and Spudich, 1998). This interaction decreases the lifetime of the M and O intermediates. The latter indicates that the interaction with HtrII accelerates deprotonation of Asp^{75}, which has been slowed, perhaps because of the absence of a proton-releasing group. (In *p*pR the residue analogous to Glu^{194} (Balashov et al., 1997; Dioumaev et al., 1998) is absent and the O intermediate has a very long lifetime.) If proton transfer from the Schiff base to Asp^{75} and elimination of the Schiff base-counterion electrostatic interaction is a prerequisite for the generation of the *p*pR signaling state (Spudich et al., 1997), then the M → *p*pR′ photoreaction should deactivate this signaling state by inducing fast reverse proton transport from the counterion back to the Schiff base. If signal generation is associated with the M or O decay, for instance, involving proton transport from the counterion to Htr during the O decay, then the photoreversal of M back to *p*pR will prevent this. One would expect that absorption of two quanta, a 500-nm quantum by *p*pR and a second 380-nm quantum by M, would result in no signal at all. The photoconversion of M would cause a decrease in the quantum yield of *p*pR similar to a decrease in the quantum yield of proton transport in bR (Ormos et al., 1978; Litvin et al., 1981). On the other hand, if signal generation is associated directly with the M formation (with the proton transport from the Schiff base to the counterion), then absorption of a quantum by M and photoconversion of M may result in a signal, opposite to the one generated upon excitation of initial *p*pR, as observed for sRI and its photoproduct M (Hoff et al., 1997). Further studies are needed to clarify the exact mechanism of coupling of the photocycle reactions with signal generation in *p*pR. Photoconversion of the M intermediate provides a useful tool for testing different scenarios.

Protein absorption changes during M formation and M photoconversion

Substantial light-induced changes of Trp environment and hydrogen bonding might be a part of a signal transduction mechanism, as has been specifically suggested for Trp^{126} and Trp^{265} in rhodopsin to meta-II transition (Lin and Sakmar, 1996; Kochendoerfer et al., 1997). Several tryptophan and tyrosine residues constitute or are close to the chromophore-binding site in bacteriorhodopsin: Tyr^{57}, Tyr^{83}, Trp^{86}, Trp^{182}, Tyr^{185}, Trp^{189} (Grigorieff et al., 1996; Belrhali et al., 1999; Luecke et al., 1999b). Some of them undergo substantial conformational changes and changes in hydrogen bonding upon formation of M (Luecke et al., 1999a). These residues are conserved in *p*pR and other retinal proteins of halobacteria (Seidel et al., 1995; Shimono et al., 1998; Mukohata et al., 1999). Formation of the M intermediate in *p*pR is accompanied by an increase in absorption at 298 and 287 nm. The amplitude of absorption changes at 287 nm corresponds to changes in extinction of ~2200 cm^{-1}/l mol (which is ~40% of the maximum extinction of a tryptophan residue). The 298-nm and 287-nm peaks can be caused by a small redshift of the absorption band of a tryptophan residue(s) and perhaps an increase in extinction due to a transition into a less polar environment or to changes in the electrostatic field (Balashov et al., 1991a; Wu et al., 1991) produced by a proton transport from the Schiff base to Asp^{75}. Similar (but more intensive) peaks at 297 and 288 nm are observed upon formation of the M intermediate in bacteriorhodopsin (Fig. 2 *B* and earlier spectra obtained under similar conditions; Sabés et al., 1984; Roepe et al., 1987). These peaks were attributed to perturbation of the environment of two to four tryptophan residues (Sabés et al., 1984). Studies of the time-resolved light-induced changes at 296 nm in the tryptophan mutants of bR led to identification of Trp^{182} as the residue responsible for

the 296-nm peak in the M minus bR spectrum (Wu et al., 1991). The nature of the shallow minimum at 272 nm in the spectrum of M minus bR is not quite clear. Elimination of hyperchromic interactions is a possible cause for the decrease in absorbance around 280 nm (Lewis et al., 1997). It may also partially originate from a redshift of a Trp residue (Roepe et al., 1987). This minimum is absent in the M minus *p*pR difference spectrum. The origin of the differences in the light-induced changes in UV that accompanied formation of the M intermediates in *p*pR and bR needs further investigation. It may be caused by differences in aromatic residues (for example, Trp^{137} in bR is replaced with Phe in *p*pR) or their involvement in the light-induced structural changes and proton transport. In bacteriorhodopsin the formation of the M intermediate not only includes proton transport from the Schiff base to the counterion but is also coupled to the conformational changes in the extracellular channel, leading to a proton release from a complex of residues, including Glu^{204}, Glu^{194}, and water molecules (Balashov et al., 1997; Dioumaev et al., 1998). In *p*pR, a carboxylic residue analogous to Glu^{194} is absent (is replaced with Pro^{183}), and proton release does not take place upon M formation but occurs during O decay (Sasaki and Spudich, 1999; Iwamoto et al., 1999b) and is accelerated in the presence of HtrII (Sasaki and Spudich, 1999). It is possible that these differences in coupling of the M intermediate with the proton transfer reactions in bR and *p*pR are relevant to the different perturbations of aromatic residues upon M formation in these two pigments.

In conclusion: We have found that the M intermediate of the photochemical cycle of *pharaonis* phoborhodopsin (*pharaonis* sensory rhodopsin II) is photoconvertible. The phototransformation of M involves several intermediate states and results in the fast light-induced reprotonation of the Schiff base and reformation of the initial pigment (or a spectrally isochromic species). Photoconversion of M comprises an additional pathway in halobacterial photoreception and may be used as a tool for the elucidation of the mechanism of signal transduction by phoborhodopsin.

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