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Biochimica et Biophysica Acta 1514 (2001) 152–158

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Selective reaction of hydroxylamine with chromophore during the photocycle of *pharaonis* phoborhodopsin

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Received 12 March 2001; received in revised form 18 June 2001; accepted 26 June 2001

Abstract

Phoborhodopsin (pR; also called sensory rhodopsin II, sRII) is a receptor of negative phototaxis of *Halobacterium salinarum*, and *pharaonis* phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, psRII) is a corresponding protein of *Natronobacterium pharaonis*. These receptors contain retinal as a chromophore which binds to a lysine residue via Schiff base. This Schiff base can be cleaved with hydroxylamine to loose their color (bleaching). In dark, the bleaching rate of *p*pR was very slow whereas illumination accelerated considerably the bleaching rate. Addition of azide accelerated the decay of the M-intermediate while its formation (decay of the L-intermediate) is not affected. The bleaching rate of *p*pR under illumination was decreased by addition of azide. Essentially no reactivity with hydroxylamine under illumination was observed in the case of D75N mutant which lacks the M-intermediate in its photocycle. Moreover, we provided illumination by flashes to *p*pR in the presence of varying concentrations of azide to measure the bleaching rate per one flash. A good correlation was obtained between the rate and the mean residence time, MRT, which was calculated from flash photolysis data of the M-decay. These findings reveal that water-soluble hydroxylamine reacts selectively with the M-intermediate and its implication was discussed.

Keywords: Bacteriorhodopsin; Bleach of chromophore; M-Intermediate; *Pharaonis* sensory rhodopsin II; *Natronobacterium pharaonis*

1. Introduction

Halobacterium salinarum has at least four retinal proteins: bacteriorhodopsin (bR) [1,2], halorhodopsin (hR) [3–5], sensory rhodopsin (sR or sRI) [6–8] and phoborhodopsin (pR or sensory rhodopsin II, sRII) [9–11]. The former two work as light-driven ion pumps and the latter two as photoreceptors of this bacterium. sR (sRI) acts as a receptor of positive phototaxis and its long-lived photointermediate absorbing 373 nm maximally is a receptor of negative phototaxis. pR (sRII) is a photoreceptor of negative phototaxis whose maximum action locates at approx. 500 nm. Using these three photosystems, this bacterium is attracted to the longer wavelength light (>520 nm) and avoids the shorter wavelength light which contains harmful near UV light.

We [12–15] and Engelhard et al. [16–19] succeeded in the purification of a pR-like protein from *Natro-*

Abbreviations: CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; DM, *n*-dodecyl-β-D-maltoside; D75N mutant, mutant in which Asp75 is substituted by Asn; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MRT, mean residence time; *p*pR, *pharaonis* phoborhodopsin; *p*pRM, M-intermediate of *p*pR; *p*pRO, O-intermediate of *p*pR

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0005-2736/01/$ – see front matter © 2001 Published by Elsevier Science B.V.
PII: S0005-2736(01)00380-7

nobacterium pharaonis and characterized the protein in great detail, because in a solubilized state it is much more stable than pR. We termed it *pharaonis* phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sRII). Recently, the functional expression of *p*pR in *Escherichia coli* was achieved [20], which provides large amounts of the protein and permits more detailed investigation.

Retinal as a chromophore binds to a lysine residue of these proteins via Schiff base. The Schiff base is susceptible to reaction with a water-soluble reagent, hydroxylamine [21], resulting in the bleaching of the pigment protein and producing retinal oximes. Bleaching of bR is enhanced by >2 orders of magnitude under illumination [22], implying that hydroxylamine reacts with photointermediate(s). This enhanced reactivity was explained by the change of water accessibility accompanied with protein conformational change during the photocycle. Thus, reactivity of Schiff base with hydroxylamine during the photocycle is thought to be a good probe for the environmental change around Schiff base.

Reactivity of *p*pR chromophore with hydroxylamine was reported in an earlier study [23], which focused on the retinal configuration of the ground state and intermediates. The present paper focused on which intermediate is attacked by hydroxylamine to lead to the bleach. We concluded that hydroxylamine reacts with the M-intermediate of *p*pR (ppR$_M$), which might suggest that a distinct environmental change around the chromophore of *p*pR occurs during the photocycle and that the environment becomes hydrophilic at the M-intermediate.

2. Materials and methods

2.1. Sample preparations

The expression of histidine-tagged recombinant *p*pR in *E. coli* BL21(DE3) and its purification were described elsewhere [24]. The Kunkel method [25] was used to prepare the D75N mutant.

2.2. Reaction of wild and D75N mutant of ppR with hydroxylamine under steady illumination

Hydroxylamine reactions were carried out at pH 7.0 in a mixture of six buffers (containing citric acid, MES, HEPES, MOPS, CHES and CAPS, whose concentrations were 10 mM each), 0.1% DM (*n*-dodecyl-β-D-maltoside), 400 mM NaCl and 10 mM hydroxylamine. The mixture of six buffers has almost equal buffer capacity for whole pH ranges. Samples were irradiated with a green light (with an interference filter of 506 nm, KL-50, Toshiba, Tokyo) from a 1 kW slide-projector lamp (Rikagaku Seiki, Tokyo). A hot mirror was placed in front of the projector lamp to remove heat radiation. Absorption spectra and time courses of absorbance changes at 500 or 520 nm were monitored by a spectrophotometer (V-560, Jasco, Tokyo).

2.3. Reaction of ppR with hydroxylamine under pulse illumination

Reactions were carried out under the same conditions as those of the steady illumination except for the hydroxylamine concentration of 50 mM. Samples were provided every 25 s by pulse illumination with a Xe-flash lamp (duration 250 μs) through an interference filter (KL-50, Toshiba). The bleaching was monitored by the absorbance at 500 nm, and the extent of bleaching was obtained as a function of the number of flashes.

2.4. Flash photolysis spectroscopy and calculation of the mean residence time (MRT)

The apparatus and the procedure of the flash photolysis were essentially the same as described previously [12]. The M-decay was monitored at 350 nm. The mean residence time, MRT, of ppR$_M$ was defined as

$$\mathrm{MRT} = \int_0^\infty t\, C\mathrm{d}t \Big/ \int_0^\infty C\mathrm{d}t$$

where C represents the time-dependent flash-induced ppR$_M$ concentration of flash photolysis data. Calculation of MRT was done numerically using Excel (Microsoft Japan, Tokyo) as follows: the flash photolysis data were taken until ppR$_M$ disappeared completely and the number of data points acquired was 450. MRT was calculated by replacement of the integral with a summation:

$$\mathrm{MRT} = \prod_{i=0}^{449} t_i C_i \delta t / \prod_{i=0}^{449} C_i \delta t = \prod_{i=0}^{449} t_i C_i / \prod_{i=0}^{449} C_i$$

where t_i and C_i are the time after the flash of the ith data point and the absorbance at t_i, and δt is the period between successive data points.

3. Results

3.1. Light-enhanced bleaching of wild-type ppR

In the dark, the bleaching reaction of the wild-type *p*pR proceeded very slowly (a rate of 0.0023/min at 20°C, data not shown) as is consistent with a previous paper [23], and illumination accelerated the bleaching. Fig. 1 shows the change in the absorption spectrum of the wild-type *p*pR, where the hydroxylamine was 10 mM and pH was 7.0. A decrease in the absorbance at 500 nm and a concomitant increase in the absorbance at 360 nm were observed. These two are proportional with the isosbestic point of 400 nm, implying that the chromophore of *p*pR reacts with hydroxylamine and produces retinal oximes which have an absorbance maximum at 360 nm. Using 33 600 M^{-1} cm^{-1} as the extinction coefficient of retinal oxime [26], the extinction coefficient of *p*pR was calculated to be 42 000 M^{-1} cm^{-1}, which is very close to the value reported previously [19]. Illumination is required for prompt bleaching and the rate was 0.39/min (see Fig. 3), which is about 170 times as fast as that in the dark, implying that hydroxylamine reacts with photointermediates of *p*pR.

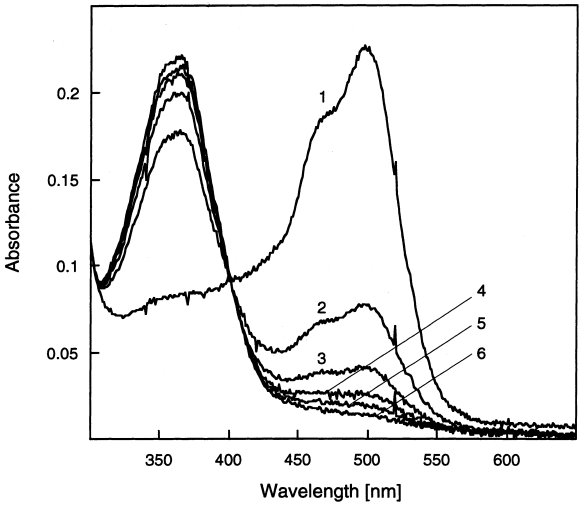

Fig. 1. Spectral changes caused by the reaction with hydroxylamine under illumination. The spectra (curves 1–6) were recorded at 0, 5, 10, 15, 20 and 30 min after addition of hydroxylamine (10 mM), respectively. Reaction mixture was irradiated with green light (506 nm at an intensity of 35 W/m^2. *p*pR of 5 μM was suspended in buffer of pH 7.0 (for composition see Section 2) at 20°C.

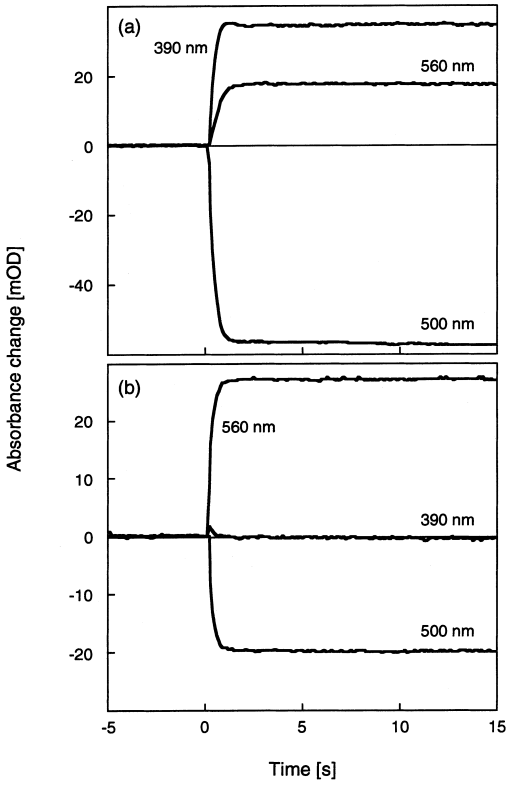

Fig. 2. Comparison of photo-steady-state concentrations of photointermediates in the absence (a) and presence of 50 mM azide. Absorbance changes at 390 and 560 nm mainly monitor the accumulation of $p\mathrm{pR_M}$ and $p\mathrm{pR_O}$, respectively, and those at 500 nm represent the decrease in the ground state pigment. Samples were started to be irradiated with green light (506 nm at an intensity of 35 W/m^2) at 0 s of the abscissa. *p*pR of 5 μM was suspended in buffer of pH 7.0, and the temperature was 20°C.

The photocycle of *p*pR after a millisecond time range is as follows: $p\mathrm{pR} \rightarrow p\mathrm{pR_M} \rightarrow p\mathrm{pR_O} \rightarrow p\mathrm{pR}$ where $p\mathrm{pR_O}$ stands for the O-intermediate. Addition of azide increased markedly the M-decay rate while neither M-formation nor O-decay changed [27]. It is anticipated, therefore, that in the presence of azide, under the photo-steady state, the concentration of $p\mathrm{pR_M}$ should decrease markedly while that of $p\mathrm{pR_O}$ should increase. The steady-state concentra-

tions of ppR$_M$ and ppR$_O$ formed by illumination (506 nm) were measured in the presence or absence of azide (50 mM), and the results are shown in Fig. 2. The concentration changes in ppR$_M$ and ppR$_O$ were monitored at 390 and 560 nm, respectively. At time 0 the continuous illumination was started to be provided, and in 10 s the photo-steady state was attained where the accumulation of ppR$_M$ and ppR$_O$ was observed. The absorbance decrease at 500 nm was mainly due to the decrease in the ground-state ppR, although absorptions of ppR and ppR$_O$ overlap each other. Fig. 2a shows data in the absence and Fig. 2b in the presence of 50 mM azide. As was expected, the photo-steady-state concentration of ppR$_M$ decreased appreciably to almost zero in the presence of azide, while azide increased the ppR$_O$ concentration.

Fig. 3 shows the effect of azide on the bleaching rate under continuous illumination (506 nm), where closed circles represent data in the absence of azide, and open circles those in the presence of 50 mM azide. Unbleached (alive) concentrations of ppR were estimated from the absorbance at 500 nm and plotted in a logarithmic scale against the duration of the illumination. The bleaching reaction followed first order kinetics and a rate constant of 0.39/min was obtained in the absence of azide. Azide slowed down the bleaching rate to 0.027/min. As described above, hydroxylamine reacts with photointermediates, and azide induces an increase in the photo-steady-state concentration of ppR$_O$ but a decrease in ppR$_M$ concentration. These findings suggest strongly that hydroxylamine reacts with ppR$_M$.

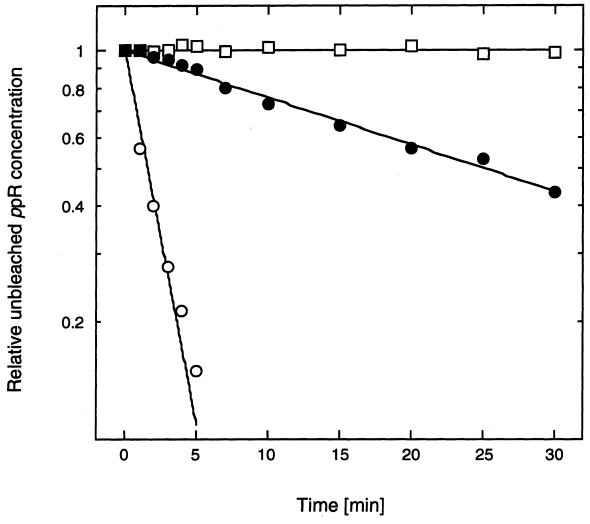

Fig. 3. Bleaching kinetics of the wild-type and D75N mutant of ppR under continuous illumination. ○, relative concentration of unbleached (alive) wild-type ppR in the absence of azide; ●, wild-type ppR in the presence of 50 mM azide; □, D75N in the absence of azide. Concentrations of unbleached (alive) pigment were estimated from the absorbance at 500 nm (for wild-type) and 520 nm (for D75N). The concentration of the pigments before illumination was taken as 1, and they were 5 μM for the wild-type and 3 μM for D75N. Here, the extinction coefficient was taken as 42 000 M^{-1} cm^{-1}, as determined in the text. Samples were irradiated with green light (506 nm at an intensity of 70 W/m^2). The buffer contained 10 mM hydroxylamine (for composition see Section 2). Temperature was 20°C.

Asp75 of ppR which is the corresponding residue to Asp85 of bR [17] acts as a proton acceptor from the protonated Schiff base: at ppR$_M$, the Schiff base is deprotonated and the proton is transferred from the Schiff base to unprotonated Asp75 [18,28]. The D75N mutant, in which Asp75 was replaced by Asn, therefore lacks the M-like intermediate and gives only one red-shifted photointermediate (O-like intermediate) in its photocycle in the millisecond time range [29]. We measured the reactivity of the chromophore of D75N with hydroxylamine under continuous illumination, and the results are shown in Fig. 3 (open squares). In the case of the D75N mutant, unbleached (alive) D75N was estimated from the absorbance at 520 nm where its absorption maximum locates [29,30]. The bleaching rate of this mutant was so slow as to be 0.00034/min, which is about 10-fold slower than that of the wild-type in the dark even though D75N was illuminated. This finding on D75N yields additional evidence that ppR$_M$ reacts with hydroxylamine.

As shown in Fig. 2, the photo-steady-state concentration of ppR$_M$ was almost zero in the presence of azide. On the other hand, the bleaching rate was slow but not zero (Fig. 3). This apparent discrepancy might come from the experimental condition of continuous illumination; although the steady average concentration of ppR$_M$ is almost zero, every ppR molecule, under continuous illumination, converts transiently to ppR$_M$ which can react with hydroxylamine. The continuous illumination was then changed to pulsed light. The results obtained are shown in Fig. 4, where the relative concentration of unbleached (alive) ppR monitored with 500 nm absorbance is plotted as a function of the number of

flashes delivered to the wild-type *p*pR in the absence and presence of 50 mM azide. It is clear that a greater number of photocycles (flashes) are required for bleaching in the presence of azide than in its absence. We further investigated the relation between bleaching rate and the existence probability of $p\mathrm{pR_M}$ during a single photocycle turnover. For the estimation of the existence probability, MRT, as defined in Section 2, was introduced. We performed the same experiments as in Fig. 4 under varying concentrations of azide, and the bleaching rate per one flash was evaluated. The period of successive flashes employed was 25 s because a singe photocycle completed in about 5 s. During the darkness between successive flashes, the reaction proceeds to some extent. This correction was done. Although it was small, we could not neglect it for the data of high azide concentration. The results are depicted in Fig. 5, where both bleaching rate per one flash and MRT are plotted against the azide concentration. The inset reveals a good proportionality between MRT and the bleaching rate per one flash. This result gives quantitative evidence that $p\mathrm{pR_M}$ reacts with hydroxylamine.

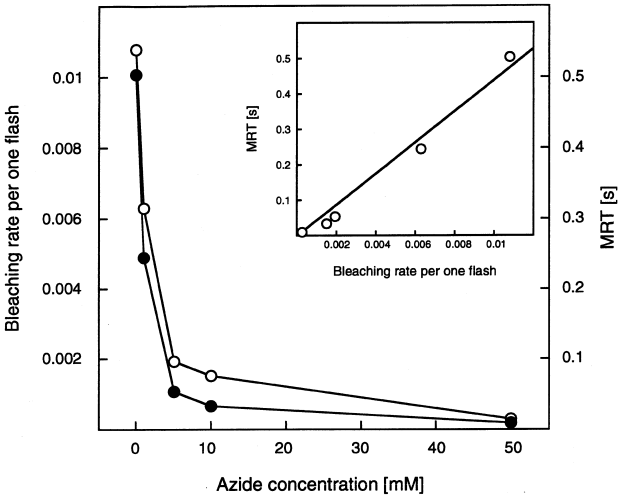

Fig. 5. Relationship between flash light-induced bleaching rate (○) and MRT of $p\mathrm{pR_M}$ (●) under varying concentrations of azide. Bleaching rates per one flash were calculated from the slope of the plots similar to Fig. 4, and corrected by subtracting the bleaching rate in the dark (flashes were not provided during measurement, data not shown). It is noted that bleaching rate in the dark cannot be neglected because more than 100 min were required to achieve the measurement of each azide concentration. Values of MRT were calculated from flash photolysis data measured at 340 nm. *p*pR (5 μM) was suspended in a buffer described in the text and pH was 7.0. Temperature was 20°C. (Inset) A good proportional relation between MRT and bleaching rate per one flash.

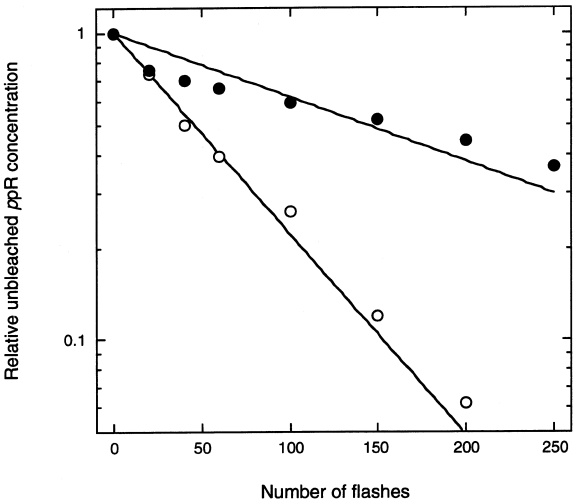

Fig. 4. Bleaching kinetics of the wild-type *p*pR in the absence (○) and presence (●) of 50 mM azide under pulsed illumination. Samples were irradiated by flash light every 25 s and absorbance changes at 500 nm were monitored. *p*pR of 5 μM was suspended in a buffer described in Section 2 and its concentration before illumination was taken as unity. Temperature was 20°C.

4. Discussion

The water-soluble reagent hydroxylamine reacts with Schiff base of retinoid proteins and produces retinal oxime [21]. It was reported that the bleaching reaction is enhanced by >2 orders of magnitude under illumination in bR [22]. Therefore, hydroxylamine mainly reacts with photointermediate(s) in the bR photocycle, reflecting the change of accessibility of hydroxylamine from the outer milieu to the active center of the protein. Early work on bR attributed this light-enhanced reaction to the M-intermediate [31,32], which is relatively long-lived and is known to be associated with deprotonation of the Schiff base and with substantial changes in protein structure. However, this hypothesis was seriously questioned by Subramaniam et al. [22], who studied the reaction in a series of bR mutants solubilized in lipid/detergent micelles. No correlation was found between the efficiency of the light-induced reaction and the presence or absence (or the lifetime) of the

M- and subsequent N- and/or O-intermediates. This led to the conclusion that the reaction occurs as a consequence of a conformational change caused by the light-catalyzed all-*trans*→13-*cis* isomerization, but before deprotonation of the Schiff base at the M-state. The L-intermediate, the precursor of M, was suggested as the species that is reactive to hydroxylamine.

On the other hand, this paper dealing with *p*pR showed that *p*pR$_M$ reacts with hydroxylamine. The possibility that *p*pR$_L$ (L-intermediate of *p*pR) is attacked by hydroxylamine like bR may be very small. The first reason is that azide influences mainly the *p*pR$_M$ decay in the millisecond to second time range of the *p*pR photocycle [27]. Two thoughts have been proposed for the mechanism of this azide effect: one is that azide binds near D85 of bR and creates hydrogen networks in the cytoplasmic channel [33], and the other is a shuttle mechanism, details of which are described in [27,34]. Apart from the mechanism, azide affects mainly the *p*pR$_M$ decay [27]. Therefore, it is very probable that the lifetime (or MRT) of *p*pR$_M$ is influenced mostly by azide. The second reason is the quantitative test revealing a good correlation between MRT and bleaching rate per one flash (inset of Fig. 5).

The L-intermediate of bR which is attacked by hydroxylamine has a protonated Schiff base. *p*pR$_M$ has a deprotonated Schiff base. It is reported that the ground state of sR (sRI) is bleached relatively easier than bR with hydroxylamine in the dark (N. Hazemoto, Ph.D. dissertation to Hokkaido University, 1983), and sRI has a protonated Schiff base. Although the rates were very small, the rate of the wild-type *p*pR in the dark is one order larger than that of D75N under illumination; both Schiff bases are protonated. These facts suggest that the reactivity of the Schiff base is influenced greatly not by the protonated state of the Schiff base but by the local environment of the chromophore. Because hydroxylamine is water-soluble, the susceptibility of this reagent might be regulated by water accessibility around the chromophore.

Proton uptake occurs at the *p*pR$_M$ decay and an electrogenic proton transport from the cytoplasmic to the extracelluar space is observed [35]. This suggests that at the *p*pR$_M$ state the cytoplasmic channel of *p*pR may open, leading to an increase in water accessibility to Schiff bases from the outer milieu, which results in the increase in the reactivity with water-soluble hydroxylamine. Similar to bR [36–39], helix movement of *p*pR during the photocycle was shown by electron paramagnetic resonance (EPR) spectroscopy [40]: an outward tilting of helix F is correlated with the early steps of the photocycle and sustained until the *p*pR$_O$ decays back to the ground state. Hence, the increase in the hydrophilicity described above might not be detected with the EPR technique. The reason for the low reactivity at *p*pR$_O$ is not known, but it might be related with the fact that the cytoplasmic channel might close after *p*pR$_O$ since during *p*pR$_O$ decay, proton release might occur through the extracellular channel [35].

The cytoplasmic channel of *p*pR is more hydrophobic than that of bR because carboxylic or polar residues such as Asp96 and Thr46 of bR are replaced by Phe and Leu in *p*pR, respectively [17]. Thus, it is expected that the reaction of hydroxylamine with *p*pR$_M$ might not be easy, but its long life might enhance the reactivity. Further experiments using a cytoplasmic channel mutant like the F86D/L40T mutant of *p*pR [41] would be interesting.

Spudich et al. [7] described that all data of both sRI and sRII (pR) can be accounted for by the following assumption: the association of sRI with its transducer, HtrI, closes the cytoplasmic channel, and the signal transmission is done by membrane helix–helix interaction which is induced by the movement of helix of the pigment. If fact, Yan et al. showed that the sRI–HtrI complex is more resistant to hydroxylamine in the dark than transducer-free sRI [42]. If the opening of the cytoplasmic channel of *p*pR (not complex) increases the reactivity of hydroxylamine, it is expected that the binding with the transducer (*p*HtrII) may reduce the reactivity because transducer may close the cytoplasmic channel [35]. Preliminary results show that the *p*pR–tHtr (tHrt means truncated *p*HtrII; for details see [40]) complex is more resistant to hydroxylamine under illumination, but in the dark the reaction rates are almost the same for the complex and *p*pR alone. More precise experiments are now in progress.

Why does hydroxylamine selectively react with *p*pR$_M$ while it reacts before the formation of M in the case of bR? It is difficult to answer this question but it may be related to the differences between two

retinal proteins on the structural changes around the Schiff base during the photocycle. This discrepancy might be involved in the relation between structure and function of bacterial retinal proteins.

Acknowledgements

This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, and by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

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