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MINI-REVIEW

Photochemistry and Photoinduced Proton-Transfer by *Pharaonis* Phoborhodopsin

N. Kamo*, K. Shimono, M. Iwamoto, and Y. Sudo

Laboratory of Biophysical Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Sapporo 060-0812, Japan; fax: +81-11-706-4984; E-mail: nkamo@pharm.hokudai.ac.jp

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Abstract—Phoborhodopsin (pR or sensory rhodopsin II, sRII) is a photoreceptor of the negative phototaxis of *Halobacterium salinarum*, and *pharaonis* phoborhodopsin (*p*pR or *pharaonis* sensory rhodopsin II, *p*sRII) is a corresponding protein of *Natronobacterium pharaonis*. The photocycle of *p*pR is essentially as follows: *p*pR(498) → *p*pR$_K$(~540) → *p*pR$_{KL}$(512) → *p*pR$_L$(488) → *p*pR$_M$(390) → *p*pR$_O$(560) → *p*pR (numbers in parenthesis denote the maximum absorbance). The photocycle is very similar to that of bacteriorhodopsin, but the rate of initial pigment recovery is about two-orders of magnitude slower. By low-temperature spectroscopy, two K-intermediates were found but the L intermediate was not detected. The lack of L indicates extraordinary stability of K at low temperature. *p*pR$_M$ is photoactive similar to M of bR. The ground state *p*pR contains only all-*trans* retinal whereas *p*pR$_M$ and *p*pR$_O$ contain 13-*cis* and all-*trans*, respectively. *p*pR has the ability of light-induced proton transport from the inside to the outside. Proton uptake occurs at the formation of *p*pR$_O$ and the release at its decay. *p*pR associates with its transducer and this complex transmits a signal to the cytoplasm. The proton transport ability is lost when the complex forms, but the proton uptake and release still occur, suggesting that the proton movement is non-electrogenic (release and uptake occur from the same side). The stoichiometry of the complex between *p*pR and the transducer is 1 : 1. *p*pR or pR has absorption maximum at ~500 nm, which is blue-shifted from those of other archaeal rhodopsins. The molecular mechanism of this color regulation is not yet solved.

Key words: phoborhodopsin, photocycle, photoinduced proton transfer

Halobacteria contain four retinal proteins (archaeal rhodopsins) which are bacteriorhodopsin (bR) [1-3], halorhodopsin (hR) [4, 5], sensory rhodopsin I (sRI) [6, 7] and phoborhodopsin (pR; also called sensory rhodopsin II, sRII) [8, 9]. The former two are light-driven ion pumps; bR works as an outward proton pump and hR as an inward halogen ion pump. The latter two are photoreceptors of this bacterium. Three photosystems (PS) of *Halobacterium salinarum* (former *halobium*) were pointed out [10], and they are PS570 (or PS565), PS370, and PS470 where the numbers represent the maximum wavelength of the halobacterial photoresponses. PS570 causes the cells to be attracted to green-orange light whereas PS370 and PS470 cause avoidance behavior (negative phototaxis) from near UV blue light and from blue-green light, respectively. Spudich and Bogomolni [6] proved that the photoreceptor of PS570 is the ground state of sRI (absorption maximum λ_{max} of sRI is 587 nm; see [11] for the difference between 570 (565) and 587 nm maxima). It was also shown that a long-lived photointermediate of sRI whose absorption maximum is 373 nm is the receptor of PS370 [12, 13]. What is the receptor of PS470? Takahashi et al. [8] isolated a mutant that showed only negative phototaxis whose action maximum is located at ~475 nm. The retinoid receptor of this negative phototaxis system was named phoborhodopsin (pR; also sensory rhodopsin II, sRII) whose absorption maximum was 487 nm [14, 15]. Similar reports were published later from other laboratories [16-18].

The primary structure of this pigment was solved by Zhang et al. [19]. sRI and pR (sRII) transmit their signals through integral membrane transducer proteins named HtrI and HtrII, which are considered to form a signaling complex firmly with respective receptors [7]. By these signaling systems, these bacteria move toward longer wavelength light (λ > 520 nm) where bR and hR work, while

Abbreviations: bR) bacteriorhodopsin; DM) *n*-dodecyl-β-maltoside; hR) halorhodopsin; pR) phoborhodopsin (sensory rhodopsin II, sRII); *p*pR) *pharaonis* phoborhodopsin (*pharaonis* sRII, *p*sRII); *p*pR$_K$, *p*pR$_L$, *p*pR$_M$ and *p*pR$_O$) K-, L-, M-, and O-like intermediate of *p*pR, respectively; sR) sensory rhodopsin; λ_{max}) absorption maximum; FTIR) infrared Fourier spectroscopy.

* To whom correspondence should be addressed.

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they avoid shorter wavelength light ($\lambda < 520$ nm), which contains harmful near-UV light [14].

The publications on the study of pR from *H. salinarium* (*s*pR, *s*sRII) were not so many because its purification was not achieved at that time (see below) and because the amounts of the protein in the cell membrane were very small. Nevertheless, several investigations were performed. On millisecond time scale at room temperature, two photointermediates were found which corresponded to M- and O-intermediates of bR [14]. The presence of another intermediate, which corresponded to K intermediate of bR, was shown by low-temperature spectrophotometry [20, 21]. At present, the expression system of *s*sRII has been achieved and the pigment was purified to investigate the primary events in its photocycle [22]. It was reported that a haloalkaliphilic bacterium (*Natronobacterium pharaonis*) had retinal pigments [23]. The absorption spectrum of one of the pigments was very similar in shape to that of pR except for a 10-nm red shift (λ_{max} at about 500 nm). In addition, it was shown that this pR-like pigment in *N. pharaonis* worked as a photoreceptor of the negative phototactic response of this bacterium [24, 25]. We named this pR-like pigment *pharaonis* phoborhodopsin (*p*pR), which is also called *pharaonis* sensory rhodopsin II (*p*sRII). We succeeded in its purification with small amounts of impurities (maybe from heme proteins) and studied its photochemistry [26]. At almost the same time, Scharf et al. [27] also investigated the photochemical conversions of *p*pR and described the stability of *p*pR in lower salt concentration while *s*sRI loses its activity gradually in low salt concentration [28]. The primary structure of *p*pR (*p*sRII) was given by Seidel et al. [29]. Later we developed an expression system of *p*pR in *Escherichia coli* cells [30], and using this system, various mutant proteins were prepared for the characterization of *p*pR. In the present mini-review, we describe the photochemistry, the photoinduced proton transfer, and the color regulation of *p*pR. Results described here are mainly from our laboratory.

PHOTOCHEMISTRY OF WILD-TYPE *p*pR

Photochemistry was studied by low-temperature spectrophotometry [26] and by flash photolysis at room temperature [31]. Irradiation of *p*pR below −100°C produced an intermediate corresponding to the K-intermediate of bR (ppR$_K$), and this intermediate was a composite of two components. The existence of two K-like intermediates was confirmed also by a recent FTIR study using a *p*pR sample expressed in *E. coli* [32]. The original *p*pR and ppR$_K$ were perfectly photoreversible as is true also for other retinal proteins.

On warming, ppR$_K$ was directly converted to an M-like intermediate (ppR$_M$) without formation of the L-like intermediate (ppR$_L$). The temperature where the conversion occurred was −70 or −90°C. This temperature is relative high, suggesting that ppR$_K$ is relatively stable at low temperature. Molecular interpretation of the existence of two stable K's awaits further investigation. The lack of ppR$_L$ was also shown in low temperature experiments of pR [21]. Note that this does not mean the lack of a L-like intermediate of *p*pR or pR: flash photolysis experiments at room temperature [31] revealed the existence of ppR$_L$. Room-temperature flash photolysis experiments were done and we [31] proposed the following photocycle:

$$p\text{pR}(498) \rightarrow p\text{pR}_K(\sim 540) \rightarrow p\text{pR}_{KL}(512) \rightarrow p\text{pR}_L(488) \rightarrow p\text{pR}_M(390) \rightarrow p\text{pR}_O(560) \rightarrow p\text{pR}.$$

Here, ppR$_O$ is an O-like intermediate; λ_{max} are given in parentheses. This photocycle resembles that of bR except that the N-intermediate was not found. The intermediates were named in analogy to those in the photocycle of bR. The species ppR$_{KL}$ was not detected at low temperature. An intermediate corresponding to the N-intermediate was difficult to observe. Chizhov et al. [33] measured flash photolysis under various conditions (temperature and pH) with 10 nsec resolution. The proposed scheme was much more complicated than that described above. They identified eight photochemically distinct kinetic states during the photocycle and some kinetic states were composed of an equilibrium mixture of intermediates including an N-like intermediate. Large changes in both the conformation and the molar volume were reported [34] upon the formation of ppR$_K$. The quantum yield of the K-intermediate was found to be 0.51 ± 0.06 [34]. The largest difference in sensor archaeal rhodopsins (sRI, pR or *p*pR) from ion-transporting ones (bR and hR) is the slowness of the photocycle. The decay time constants of ppR$_M$ are ~20 sec^{-1} (pH 5) and 1 sec^{-1} (pH 9), and those of ppR$_O$ are 3-4 sec^{-1} which are almost independent of pH for the *n*-dodecyl-β-maltoside (DM) solubilized sample suspended in 400 mM NaCl. The salt concentration affects the photocycling rate only to a small extent. Although this large pH dependence of the M-decay was not observed by Chizhov et al. [33], this dependence [35] helped to determine the steps in the photocycle that are coupled to proton uptake and release. As in the case of M of bR, the retinal configuration of ppR$_M$ is 13-*cis* and the Schiff base is deprotonated. The ppR$_O$ has all-*trans* chromophore and protonated Schiff base [36]. The ground state *p*pR contained only all-*trans* retinal (all-*trans*, 6S-*trans* for pR [37]), meaning that there is no light-dark adaptation [38]. When 13-*cis* retinal was added to *p*pR-opsin, two phases of the absorbance change were observed with half-times $5.9 \cdot 10^3$ and 10^4 sec. The λ_{max} shifted gradually to the longer wavelength during the reconstitution with 13-*cis* retinal. After no shift was observed, only all-*trans* retinal was extracted. These observations were interpreted as [38]: 13-*cis* retinal can

bind *p*pR-opsin very slowly and λ_{max} of the 13-*cis* pigment is blue shifted from that of the all-*trans* retinal pigment by ~10 nm. After the binding, the chromophore isomerizes spontaneously to all-*trans*. On the other hand, reconstitution with all-*trans* retinal was rapid with a halftime of ~3 min [39]. This implies that the chromophore binding pocket has no space which can easily accommodate 13-*cis* retinal. This may be a reason for the lack of light-dark adaptation. Reconstitution experiments using retinal analogs showed that the space of the retinal binding site is restricted to the plane of the cyclohexenyl ring of the chromophore [39].

Photoactivity of M. In bR, photoexcitation of the M-intermediate causes fast reprotonation of the Schiff base [40, 41] from the counterion Asp85 [41-44] and transformation of the pigment back to its initial state through a non pumping pathway. Phototransformation of M involves the formation of two primary photoproducts, P421 and P433 [42, 44], also called M′ [45], and several subsequent thermal intermediates, which are in turn photoactive [44]. The analogous photo-transformation of $p\text{pR}_M$ was found [46]. Several new intermediates are formed during phototransformation of $p\text{pR}_M$ back to the initial *p*pR as was revealed by a low temperature study [46]. The scheme is as follows: $p\text{pR}_M(390) \rightarrow \text{M}'(404) \rightarrow p\text{pR}'(496) \rightarrow p\text{pR}'(504) \rightarrow p\text{pR}$. Reversible phototransformations $p\text{pR} \leftrightarrow p\text{pR}_M$ were observed at −60°C. They were accompanied by the perturbation of tryptophan(s) and probably tyrosine(s) residues, as reflected by changes in the UV absorption band [46]. The phototransformations of $p\text{pR}_M$ at room temperature is now being investigated.

Acceleration of M decay by azide. The decay of M was strongly accelerated by addition of azide, similar to that in the D96N mutant of bR in which Asp96 was substituted by Asn [47] and M-decay rate was very slow due to the lack of an internal proton-donating group (Asp96) to the deprotonated Schiff base of M. In *p*pR, the analogous donor is also absent: a residue corresponding to Asp96 of bR is Phe86, which probably explains at least partially the slow decay of $p\text{pR}_M$. The addition of azide (500 mM) at pH 7.0 increased the rate of $p\text{pR}_M$-decay by 300-fold [48]. Arrhenius analysis revealed decreases in the activation energy and a further decrease in the activation entropy. In other words, the increase in the rate originates from the decrease in the activation energy while the entropy (the frequency factor) has a negative effect. This is supposed to be related to the narrow chromophore binding pocket [39] as described above.

PHOTOCHEMISTRY OF MUTANT *p*pR

In the M-state of bacteriorhodopsin, the Schiff base is deprotonated and the decay of M coincides with the reprotonation of the Schiff base by the hydrogen-bonding chain that includes Asp96 and Thr46. In *p*pR these amino acids are replaced by Phe(F86) and Leu(L40) [29]. The absence of these internal proton donors in *p*pR may account for the slow decay of $p\text{pR}_M$. The M-decay of F86D mutant was not significantly accelerated. However, in a double mutant (F86D/L40T) in which the internal proton donor and possibly hydrogen-bonding network was restored, the M-decay was accelerated as much as approximately 36-fold at pH 5.0 and 130-fold at pH 7.0 [49]. On the other hand, a mutant in which O-decay becomes faster than the wild-type has not been found yet.

Upon the formation of M of bR, the proton from the Schiff base is transferred to the proton acceptor, Asp85. The corresponding Asp of *p*pR is Asp75 [29]. This residue is considered, to be a proton acceptor from the protonated Schiff base [50]. Therefore, the D75N mutant does not form $p\text{pR}_M$ upon illumination, and this mutant has along-lived K-like intermediate [51]. This mutant, however, shows a photoinduced absorption band whose λ_{max} is 330 nm, and this band appears also in the photocycle of the wild-type *p*pR when careful inspections of the flash-induced difference spectra were done [52]. If an intermediate responsible for this absorption band is another M-intermediate judging from the location of λ_{max}, a question arises which residue works as the proton acceptor. Another possibility is that this absorption band is a β-band of an intermediate. The decay constant of this band does not match that of M and O. Therefore, it might be a β-band of the N intermediate which is hard to detect as described above. The β-band of N of bR has a maximum at 330 nm (see review [53] and papers cited therein). The protonation state of Asp75 influences the λ_{max} of *p*pR [33], as is similar to bR; this Asp is considered to be a counterion to the Schiff base. Upon acidification in the absence of chloride, λ_{max} shifted to 522 nm. This bathochromic shift is thought to be caused by the protonation of Asp75. The D75N mutant has its λ_{max} at approximately 520 nm, supporting this mechanism of the bathochromic shift. A titration of the shift yielded a pK_a of 3.5 for Asp75. In the presence of chloride, the spectral shifts were different: with a decrease in pH, a bathochromic shift was first observed, followed by a hypsochromic shift on further acidification. This was interpreted [54] as the disappearance of a negative charge upon protonation of Asp75 being compensated by the binding of chloride, but it is worth noting that the binding of chloride required the protonation of Asp75 and of a second group other than Asp75. This was supported by the observation that in the presence of chloride, upon acidification, the λ_{max} of D75N showed a blue shift, indicating that the protonation of a proton-associable group leads to the chloride binding that gives rise to a blue shift. Identification of these groups needs further work.

LIGHT-INDUCED ELECTROGENIC PROTON TRANSPORT

The SnO_2 electrode is considered to monitor the pH change. Iwamoto et al. [55] constructed a photo-electrochemical cell composed of SnO_2/thin *p*pR solution (~50 μM)/400 mM NaCl/SnO_2. Photo-induced potential differences between two SnO_2 transparent electrodes were measured. They were caused by changes in pH close to the SnO_2 electrode surface. The signal was time-differentiated to envisage the direction of pH change and proton movement. A positive signal was due to a decrease in the local pH, i.e., proton release from *p*pR, and a negative signal was caused by the proton uptake. Immediately upon irradiation with continuous light, the transient negative on-response was observed for all pH values examined. The shape of the off-response to turning off the light was pH-dependent: at alkaline or neutral pH, a negative component was observed followed by a positive component. Another experiment on the off-response was done; the off-response was measured after the photo-steady state was attained. The shape of the off-response under varying pH well correlated with the ratio of contents of ppR_M and ppR_O at the steady state. Note that the ppR_M decay is pH-dependent. It is concluded that the proton uptake occurs during $ppR_M \rightarrow ppR_O$ and the proton release during $ppR_O \rightarrow ppR$ transitions. Illumination of *p*pR-containing membrane vesicles showed the sustained pH deflection in the medium, meaning the photo-induced membranous transport of proton whose direction is the same as that of bR [56].

Engelhard, Bamberg, and their colleagues [57, 58] using black lipid membranes or the oocyte system showed that *p*pR can transport protons upon illumination. They observed a small photocurrent at pH 5.0, and an increased current for the F86D mutant. Addition of azide increased the pump efficiency (the photocurrent) significantly. On the other hand, azide increases the ppR_M decay but has no effect on ppR_O decay, which is slower than ppR_M decay. This means that the time required for the completion of the photocycle does not change significantly by the addition of azide. Why does addition of azide increase the photocurrent but not change in the time for the photocycle completion? They proposed a hypothesis of two-photon process suggesting that O-intermediate is photoactive which accelerates the photocycling rate under constant illumination [58]. The experimental proof is not yet obtained. Sudo et al. [56] and Schmies et al. [58] showed that the photo-induced proton transport ceased when *p*pR associates with the transducer while the photocycling rate was not significantly changed when they were co-expressed in membranes [56, 59]. Light-induced proton uptake and release were still observable even when *p*pR and the transducer formed the complex [56], although the vectorial nature of the proton movements was inhibited. Therefore, proton circulation (proton release and uptake from the same side) might occur, which was observed for the pR–transducer complex by Sasaki and Spudich [9, 60]. Sudo et al. [56] interpreted their results as the closure of the cytoplasmic channel caused by the association with its cognate transducer. This had been proposed by Spudich and his colleagues for pR and sR [9, 61]. Experimental verification for this is necessary.

COLOR REGULATION

The λ_{max} of *p*pR or pR is remarkably different from those of the other three archaeal retinal proteins: bR, hR, and sR have their λ_{max} at 560-590 nm while that of *p*pR or pR is blue-shifted to ~500 nm [62]. What is the molecular mechanism for the λ_{max} of *p*pR or pR being different, although all archaeal rhodopsins are highly similar in their primary structure, especially in the chromophore binding site? According to the amino acid sequences of bR and *p*pR, retinal binding pockets of these proteins differ at only seven positions (Ile43, Ile83, Asn105, Val108, Phe127, Gly130 and Phe134 for *p*pR and Val49, Leu93, Asp115, Met113, Trp138, Ser141 and Met145 for bR, respectively). Furthermore, there exist three additional positions when the distance of a residue from the retinal is extended to 5 Å (Met109, Ala131, and Thr204 for *p*pR and Ile119, Thr142 and Ala215 for bR, respectively). To determine key residues causing different opsin-shift in *p*pR and bR, Shimono et al. [63-65] constructed *p*pR mutants in which each of the specific residues of the above sites was replaced by a corresponding residue of bR. In addition, 7- and 10-residue-substituted mutants were constructed which were expected to have the same amino acid residues as bR with respect to the retinal binding site. In spite of the amino acid arrangement around the chromophore being the same as that of bR, the red shift from the original *p*pR was not large (λ_{max}, 524 nm for the 10-substituted mutant), suggesting that the shape of the retinal binding site in *p*pR differs from that in bR, and that other structural factors are more important for the difference of λ_{max} between the two than the specific set of residues in the retinal binding pocket. High resolution structures by X-ray or cryo-electron crystallography [66, 67] may be needed to further analyze the color tuning.

FTIR [32, 50] and resonance Raman spectroscopy [68] showed that frequency derived from the C–C stretch of the polyene chain in *p*pR does not differ from that of bR. Therefore, the difference of the structure around the chromophore between *p*pR and bR may originate from the environment near the β-ionone ring [32]. Furthermore, the hydrogen bond between the Schiff base and its counter-ion is stronger than that in bR. Another spectroscopic difference of *p*pR or pR from the other three is the existence of a shoulder in the absorbance

spectrum. The origin of the shoulder was proposed by Takahashi et al. [62] to be vibrational fine structure due to the fixation of the retinal within the binding site. The β-ionone ring was suggested to be fixed [32]. This is supported by reconstitution experiments with the retinal analog described above.

FURTHER UNSOLVED PROBLEMS

As described above, there are several unsolved questions.

1. The molecular mechanism of the photo-induced proton transport of *p*pR is unknown. In bR, the proton releasing group is tentatively identified as a complex of residues including Glu204, Glu194 and water molecules. What is the proton releasing group of *p*pR? To investigate this, we are preparing mutants whose dissociable amino acids are replaced and their proton pumping activities are going to be measured. Also, FTIR study of these mutants may be necessary to check the existence of hydrogen bonding networks or the presence of functional water within the protein.

2. How can *p*pR transmit signals to the transducer? On this question, Engelhard and his group published an interesting paper using EPR (electron paramagnetic resonance) [59]. They showed that similar to bR, helix F and G of *p*pR moves on illumination. This conformational change might be transmitted to the transducer as a signal as was suggested by Spudich [61]. Sudo et al. [69] showed that *p*pR binds to its truncated transducer even in the presence of a detergent, DM with a 1 : 1 stoichiometry.

3. How do the proton-transfer reactions contribute to the helix movement and transducer activation? Is the proton-transport a side effect? Or does this proton movement induce the helix movement?

4. Why is λ_{max} of *p*pR blue-shifted? As described above, for elucidation of the color regulation, the structure, especially that of the retinal binding pocket is necessary. Spudich et al. [66] and Kunji et al. [67] succeeded in analyzing the structure, which will give an important clue to solve the mechanism of color regulation. However, their resolution was 6.9 Å, and a structure with a higher resolution might be necessary.

5. Four archaeal rhodopsins have similar structures. Seven helices constitute transmembrane portion of the protein, and a retinal chromophore is bound to a lysine residue of the seventh helix *via* a protonated Schiff base linkage. How are these archaeal rhodopsins differentiated into ion pumps and sensors? What are the molecular mechanisms for this differentiation?

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