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Photochemistry of *pharaonis* phoborhodopsin and its interaction with the transducer

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Phoborhodopsin (pR or sensory rhodopsin II, sRII; the absorption maximum of ~ 500 nm) is a retinoid protein and works as a photoreceptor of the negative phototaxis of *Halobacterium salinarum*. *pharaonis* phoborhodopsin (*p*pR or *pharaonis* sensory rhodopsin II, psRII) is a corresponding protein of *Natronobacterium pharaonis*. These sensory proteins form a complex with a cognate transducer protein in the membrane, and this complex transmits the light-signal to the cytoplasm to evoke avoidance reaction from blue-green light. Recently, the functional expression in *Escherichia coli* membrane of *p*pR was achieved, which can afford a large amount of the protein and enables mutant studies to clarify the role of various amino acid residues. A truncated transducer which can bind to *p*pR is also expressed in *Escherichia. coli* membrane. In this article, we will review properties of *p*pR mainly using observations of our laboratory; which contains photochemistry (photocycle), light-driven proton uptake, release and transport, F-helix titling during photocycle and association of the transducer.

Key words: Photocycle, Proton transport, Flash photolysis, SnO_2 electrode, Helix tilting, Photo-taxis

Halobacteria contain four retinal proteins (archaeal rhodopsins), which are bacteriorhodopsin (bR), halorhodopsin (hR), sensory rhodopsin (sRI) and phoborhodopsin (pR; also called sensory rhodopsin II, sRII) [1,2]. 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. Spudich and Bogomolni [3] proved that the ground state of sRI (absorption maximum λ_{max} of 587 nm) is a photoreceptor of the positive taxis, and that the long-lived photo-intermediate of sRI (λ_{max} of 373 nm) is a receptor of the negative phototaxis. A mutant that showed only negative phototaxis was isolated and the action maximum located at ~470 nm [1]. The retinoid receptor of this negative phototaxis was named phoborhodopsin (pR; also sensory rhodopsin II, sRII) [2]. SRI and pR (sRII) transmit their signals through integral membrane transducer proteins named HtrI and HtrII (Htr is an abbreviation of halobacterial transducer), which form a signaling complex firmly with respective receptors. By these signaling systems, these bacteria move toward longer wavelength light (λ > 520 nm) where bR and hR work, while they avoid shorter wavelength light (λ < 520 nm), which contains harmful near U-V light.

The publications on the study of pR from *H. salinarum* were not so many because its purification was not achieved at that time (although, at present, the expression system of sRII has been achieved and pigment was purified to investigate the primary events in its photocycle), and because the amounts of the protein in the cell membrane are very small. We purified a pR-like pigment with small amounts of impurities from cell membranes of haloalkaliphilic bacterium (*Natronobacterium pharaonis*) and named *pharaonis* phoborhodopsin (*p*pR) [4]. This *p*pR is very stable protein in the solubilized state with dodecyl-β-D-maltoside (DM). The primary structure of *p*pR was given by Seidel et al [5]. Later we developed a functional expression system of *p*pR in *Escherichia coli* cell membranes [6], which affords to supply a large amount of proteins and enables the mutant study so as to find out the functionally important amino acid residues. Using this expression system, the X-ray crystallographic structure [7,8] has been solved, which will open the new era in the *p*pR studies.

This article describes the recent progress of *ppR* based mainly on results reported from our laboratory. A review article by the present author was published [9].

PHOTOCHEMISTRY OF THE WILD-TYPE *p*pR

Photochemistry was studied by low-temperature spectroscopy [4] and by flash photolysis at room temperature [10]. The photocycle was proposed 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. Here, an intermediate of *p*pR_i is the intermediate similar to the i-intermediate of bR. The number in the parenthesis represents the maximum wavelength of an each intermediate. An intermediate corresponding to N-intermediate is difficult to observe. Chizhov et al. [11] measured flash photolysis under various conditions with 10 ns resolution, and proposed a scheme containing the N-intermediate that is much more complicated than that described above. This photocycle resembles that of bR except several points. They are 1) shorter absorption maximum wavelength with vibration fine structure, 2) extraordinary high thermal stability of the K-intermediate [4,12], 3) more extended structural changes in view of chromophore distortion and protein structure [12], 4) slower photocycling rate, and 5) no light-dark adaptation [13,14] (all-*trans* and 6S-*trans* retinal [7,8] of the chromophore configuration of the ground state). In addition, retinal-analog experiments [15] show the different structure around the retinal from bR.

The reason of the slow decay of *p*pR_M is due to the lack of an amino acid residue donating a proton to the deprotonated Schiff base during the M-decay. Replacement of Phe86 with Asp led to the faster M-decay, and double mutation of F86D/L40T showed the decay of as fast as that of bR [16], where the 86 position correspond to D96 of bR.

In acidic condition, the absorption maximum of bR shifts to longer wavelength (bathochromic shift), which is originated from the protonation of Asp85. Similarly, the bathochromic shift of *p*pR was observed in acidic condition. In the presence of Cl^-, however, the bathochromic shift did not occur [17]. This is interpreted by the Cl^- binding, which compensates the disappearance of a negative charge (maybe association of a carboxyl) other than D75 which is corresponding residue of D85 of bR.

The color regulation of *p*pR was investigated experimentally [18-20] and theoretically [21,22], but the satisfactory explanation might not be given yet.

LIGHT-DRIVEN ELECTROGENIC PROTON TRANSPORT

The SnO_2 electrode is considered to monitor the pH change. The off-response on the pH-change in the medium after the photo-equilibrium was investigated. At the acidic condition, the proton release was observed while, at the alkaline condition, the proton uptake followed by the release was observed. The M-decay rate is faster in an acidic than in an alkaline medium while the O-decay is pH-independent. In acidic condition, the M-decay rate is faster than that of O-decay rate while, in alkaline condition, the ratio of rate constants are reversed. Therefore, at acidic condition, the composition at the photo-equilibrium is mainly the O-intermediate while, at alkaline condition, it consists mainly of M-intermediate. Considering these, we inferred that proton uptake may occur at the M-decay and the release may occur at the O-decay [23].

Does this light-induced proton movement lead to the light-induced transmembrane transport (electrogenic transport)? This was examined using membrane vesicles: Upon illumination to vesicles the pH change in the suspending medium was observed, implying the transmembrane proton transport. More direct check of electrogenecity using lipid bilayers revealed that the activity of the electrogenic transport was much weaker than that of bR. This might be originated from the high hydrophobicity of cytoplasmic (CP) channel, which was supported by the observation that the transmembrane proton transport of F86D mutant was larger than that of the wild-type. Therefore, we concluded that at the M-decay, the proton comes to the deprotonated Schiff base from both CP and the extracellular (EP) side [24].

SPUDICH'S PROPOSAL ON THE SIGNAL TRANSDUCTION AND HELIX TILTING

Spudich proposed that disruption of the salt bridge between helices C and G which triggers the outward tilting of helix F, and that this tilting is a signal to be transferred to the cytoplasm. This is based on the accumulated observations on F- or G-helix movement of bR during the photocycling. The helix movement of F-helix of *p*pR was shown by Wegener et al. using EPR [25]. Here, we will present our own data on the F-helix opening. We prepared various cysteine mutants that contain only one cysteine at various positions, and observed the photo-induced irreversible inactivation of several mutants when SH-reagent of relatively large molecule size was present. The positions of cyteine in these mutants are located at the inner surface of F-helix. In the presence of azide that accelerates the M-decay, the irreversible inactivation was not observed. These results imply that at M-decay, the F-helix may tilt outward which makes the space where the SH-reagent can enter to react the cysteine residue. Due to the bulkiness of the reagent, the F-helix cannot return to the original position, which lead to the irreversible inactivation.

ASSOCIATION OF *pharaonis* HALOBACTERIAL TRANSDUCER (pHtrII)

As described above, ppR associates with *pharaonis* halobacterial transducer (pHtrII) in the membrane. We observed the cessation of the transmembrane proton

transport of membrane vesicles when both ppR and pHtrII were co-expressed in the halobacterial cell membrane [24]. On the other hand, membrane sheets (not enclosed vesicles) revealed still the light-induced proton uptake and release. These observations imply that the association of *p*pR with the *p*HtrII closes the CP channel of *ppR* and then the futile proton circulation at the EC side occurs [24].

It is shown that association of *p*pR with its transducer occurs even in the presence of DM, a detergent, and that the dissociation value K_d was 0.1 μM for the wild-type *p*pR (psRII)/t-Htr complex [26]. Here, t-Htr stands for a truncated pHtrII which loses the cytoplasmic regions of pHtrII, because the full length pHtrII was not expressed in *E. coli* cells. It is considered that the sensor interacts with the transducer via the helix-helix interactions within the membrane. t-Htr contains the native helix part embedded within the membrane, and hence the interaction between *p*pR and t-Htr simulates well that of pHtrII.

We observed that hydroxyl amine attacked the M-intermediate to bleach [27] and that the bleaching rate became slow when *p*pR is associated with the transducer [28]. As described above, azide accelerates the M-decay, but we observed that azide effected the M-decay of the transducer-free *p*pR by 4.6-fold than that of the ppR/t-Htr complex. These observations suggest that some conformational changes near the chromophore occur when *p*pR binds with the transducer.

M-decay rate was observed to be affected by the association with t-Htr in 400 mM NaCl containing 0.1% DM. The decay time constant was 1.66 s^{-1} for the free *p*pR while it was 0.82 s^{-1} for the complex. We titrated t-Htr with *p*pR to measure the flash-induced absorbance change. The flash photolysis traces were contributed from two components that were free *p*pR and *p*pR/t-Htr complex. The concentrations of free *p*pR and *p*pR/t-Htr were estimated from the amplitudes of α and β of the fitting equation of $\alpha\exp(-1.66t) + \beta\exp(-0.82t)$ assuming that the same extinction coefficients for the free and the complex. Using the mass action law and the mass balance equations, K_d value was estimated to be 15.2 μM and the stoichiometry ratio of *p*pR to the complex was 1.2, which means the (1:1) complex [29]. The transducer exists as a homo-dimer, and then this value indicates (2:2) of the complex. The plausible model composing of t-Htr and *p*pR was given.

The k_d value determined by Wegener et al [26] was 0.1 μM, and our value determined with flash photolysis was 15.2 μM. Why do these values differ from each other? The value of 15.2 μM may be the value for the value at M-intermediate when we consider the method. In another word, at M-state the interaction between *p*pR and the transducer becomes weak, which might be consistent with the observation of EPR that has deduced the rotation of the transducer during the photocycle [25]. Because the application of the similar method to the interaction of the O-intermediate and the transducer was not possible, we substituted an O-like intermediate of D75N mutant for the O-intermediate of the wild-type because this O-like intermediate is a sole intermediate of the photocycle of D75N after ms time range. The K_d value for the interaction between the O-intermediate and the transducer is 0.15 μM, which is similar to that in the dark. This small K_d value of the O-intermediate might be consistent with the F-helix titling that, at O-state, the helix may close while at M-state the helix may open. On the other hand, EPR data showed that the helix movement begins at the M-formation and lastes to the O-decay. It has been suggested that both M- and O-state are signaling states, which also seems contradictory to the change of K_d values described above. Further study is necessary.

Two groups [7,8] solved the X-ray crystallographic structure of *p*pR, and from the structure the importance of Tyr199 or positively charged surface patch has been pointed out for the molecular interpretation of the association. Hence, we checked the proposal experimentally, and obtained the results suggesting both are working.

More detailed experiments concerning the signal transduction mechanism are necessary and this is a further problem to be solved.

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