Light-Induced Proton Uptake and Release of *pharaonis* Phoborhodopsin Detected by a Photoelectrochemical Cell

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Phoborhodopsin (pR, also called sensory rhodopsin-II, sR-II) is a receptor for the negative phototaxis of *Halobacterium salinarum* (pR), and *pharaonis* phoborhodopsin (*p*pR) is the corresponding receptor of *Natronobacterium pharaonis*. pR and *p*pR are retinoid proteins and have a photocycle similar to that of bacteriorhodopsin. The photocycle of *p*pR in the millisecond time range includes M and O intermediates: *p*pR → M → O → *p*pR. A photoelectrochemical cell was constructed composed of SnO_2|thin *p*pR solution (∼50 μM)|400 mM NaCl|SnO_2. Photoinduced 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 examined. The shape of the off-response on turning off the light was pH-dependent: at alkaline or neutral pH, a negative component was observed followed by a positive component. The off-response was measured after the photosteady state was attained. The shape of the off-response well correlated with the ratio of contents of the M and O intermediates at the steady state. It is concluded that the proton uptake occurs during M → O and the proton release during O → *p*pR transitions.

Introduction

Halobacterium salinarum (*halobium*) has at least four retinal proteins: bacteriorhodopsin (bR),[1,2] halorhodopsin (hR),[3,4] sensory rhodopsin (sR or sR-I),[5−7] and phoborhodopsin (pR or sensory rhodopsin II, sR-II).[8−12] The former two work as light-driven ion-pumps and the latter two as photoreceptors of this bacterium. pR (sR-II) is a photoreceptor of negative phototaxis whose maximum action is located at approximately 500 nm while the maximum wavelengths of the other three proteins are about 560−580 nm. Much less is known about pR (sR-II) although the first three retinal proteins have been studied in great detail.

We[13−16] and Engelhard et al.[17−20] have succeeded in the purification of a pR-like protein from *Natronobacterium pharaonis* and have 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*sR-II). Recently, the functional expression of *p*pR in *Escherichia coli* has been achieved,[21] which provides large amounts of the protein itself and permits investigation of its photochemistry.

On light excitation, the bacterial rhodopsins react in a cyclic manner, forming a series of intermediates before returning to their original state. Although the photocycling rate is almost 2 orders of magnitude slower than that of bR,[20,22] the photochemistry of *p*pR (or pR), similar to bR, includes K-, L-, M-, and O-like intermediates.[20,22] This similarity leads us to suggest that similar to bR, the proton movement might take place during the photocycling. Actually, Engelhard et al.[23] observed the photoinduced current of *p*pR incorporated into black lipid membranes in the presence of azide which accelerates the photocycle considerably.[24] This observation implies that the ion translocation occurs through the protein. The ion translocated might be a proton, but has not yet been proved.

Koyama et al.,[25−29] Robertson and Lukashev,[30] and Wang et al.[31,32] constructed a photoelectrochemical cell composed of SnO_2/purple membrane. They measured the photoinduced electric current and observed time-differentiated signals; when illumination was provided, the transient positive on-response was observed, followed by the transient negative off-response when illumination was removed. Using this cell the following was reported: (1) the orientation of the purple membrane did not affect the polarity of the bipolar response;[27] (2) only the all-*trans*-bR gave the signal;[28] (3) the response of D96N mutant agreed with the proton movement observed by other experiments;[29,30] (4) the responses of bR mutants lacking the terminal proton-release residues in the extracellular region (Glu-204 and Glu-194)[29,33−36] was negative on-response followed by positive off-response,[29,33] which is opposite to that of the wild-type bR. The experiments using pH-sensitive dyes showed proton uptake precedes the release in these mutants,[36] and (5) when *pharaonis* halorhodospin (*p*hR) adhered to the SnO_2 electrode surface, the signal was observed only when *p*hR transported protons.[37] When halide ions were transported, responses have not been observed. These observations led us to conclude that the SnO_2/protein

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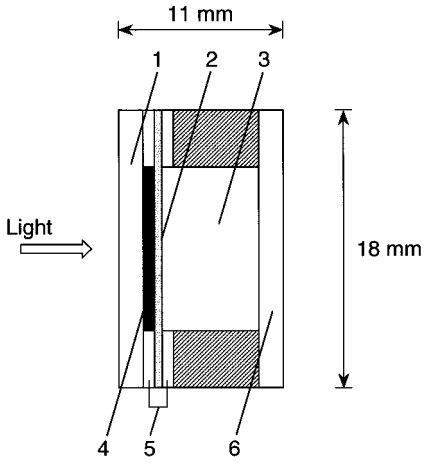

Figure 1. Structure of the photoelectrochemical cell: (1) a SnO_2 transparent electrode (diameter of 1 cm); (2) dialysis membrane; (3) 400 mM NaCl; (4) a thin layer of *p*pR solution (ca. 50 μM, 0.5 mm in thickness); (5) a silicone O-ring; (6) a counter SnO_2 electrode. The potential difference between 1 and 6 were measured with Nihon Koden Bioelectric Amplifier MEG-1200 having adjustable low-cut filters ranging from 0.08 to 150 Hz.

photoelectrochemical cell monitors the local pH change adjacent to the electrode surface.[30−32,38] Actually it was shown that the potential between the oxide electrode and a reference electrode was linear against pH of the solution.[30] Using SnO_2 electrode, the present paper shows that proton uptake by *p*pR occurs first followed by the proton release: the proton uptake may coincide with the decay of M-intermediate (formation of O-intermediate) and the release, the decay of O-intermediate.

Materials and Methods

Expression of the Recombinant *p*pR. The procedures were essentially the same as those described previously:[21] *E. coli* BL21(DE3) was used for transformation and expression of the gene. The plasmid pFEppR[21] was used for functional expression of *p*pR. The cells were grown in 2 × YT medium supplemented by ampicillin (final concentrated of 50 μg/mL).

Purification of *p*pR. At 120 min postinduction, cells were harvested by centrifugation at 4 °C, suspended in a buffer containing 50 mM Tris-Cl (pH 8.0) and 5 mM $MgCl_2$, and stored frozen at −20 °C. Thawed cells were disrupted by sonication for 5 min at 4 °C with a power of 160 W at a duty cycle of 50% (UP200H, Kubota, Tokyo). Crude membranes were collected by centrifugation (100 000*g*) for 120 min at 4 °C and washed with a low-salt buffer (20 mM NaCl, 10 mM Tris-HCl, pH 8.0). Membranes were solubilized in the dark overnight at 4 °C and in the presence of 1.5% *n*-dodecyl-β-D-maltoside (DM). *p*pR was further purified by anion exchange chromatography on DEAE-Sepharose (CL-6B, Pharmacia) equilibrated with 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 0.1% DM. The protein was eluted with a linear gradient from 50 to 200 mM NaCl. A peak fraction containing *p*pR was collected. Samples for the photoelectrochemical cell were prepared by dialysis against distilled water to remove buffer components.

Flash Spectroscopy. Apparatus and procedure were essentially the same as described earlier.[21]

Construction and Procedure of the Photoelectrochemical Cell. A schematic illustration of the cell used is shown in Figure 1. A pair of SnO_2 electrodes was used: one of them as a working electrode and the other a counter electrode. The active area of the working electrode was 1 cm of diameter. The cell was composed of SnO_2|thin *p*pR solution|400 mM NaCl|SnO_2. A thin layer of a *p*pR solution (ca. 50 μM) containing no buffer covered the working electrode and was overlaid with a dialysis membrane. This differs from the original photoelectrochemical cell made by Koyama et al.[28,29] who employed a procedure in which a purple membrane was dried on an SnO_2 electrode and adhered to the electrode surface. The working electrode adjoining the *p*pR solution and the counter electrode were set 8 mm apart and mounted into a cell, which was filled with 400 mM NaCl without buffer. The pH was adjusted by HCl or NaOH.

The light source for illumination of the above sandwich-type thin electrochemical cell was a 300 W xenon arc lamp in combination with an infrared cutoff filter and color filters (HA50, CM500, and Y50, Toshiba), which provided green light with a maximum intensity at around 530 nm. The intensity was 1.34 kW/m^2 measured with a Kettering radiant power meter (Model 4090, Yellow Springs). The two SnO_2 electrodes were connected to an amplifier (Bioelectric Amplifier MEG-1200, Nihon Koden) equipped low-cut filters ranging from 0.08 and 150 Hz. The potential difference between the two SnO_2 electrodes was also measured with an electrometer (Takeda Riken, TR8651) and the signal was almost the same as those obtained with a low-cut of 0.08 Hz, meaning that the signal obtained with 0.08 Hz low-cut was the potential change by illumination. The output of this amplifier was connected to a digital storage oscilloscope (Hewlett-Packard model 54520C) to store the signal.

As shown below, the potential difference between the two SnO_2 electrodes by illumination to *p*pR was negatively deflected. The exposure duration was changed from 6.7 ms to 1 s. With increasing duration, the magnitude of responses increases with the time and becomes saturated after 71 ms, and the signal curves were superimposed each other (data not shown). Then 1 s of illumination was usually employed.

Results

Figure 2, a and b, shows the voltage change of the cell under illumination to *p*pR. The pH was 8.8 (a) and 4.7 (b). The upper curve shows the voltage change and the lower rectangular signal indicates the period of illumination. These curves were obtained when the low-cut frequency was set to 0.08 Hz, and as described above, they represent the potential change of the working SnO_2 electrode. In the absence of the *p*pR solution, no photoresponse was observed, showing SnO_2 electrodes were inert to the illumination.

Figure 2a′ is the result obtained under the same condition of (a) except that the low-cut frequency was set at 15 Hz, and then this curve (a′) represents almost the differential of the curve (a). By this differentiating process, components of the proton movement become clearly separated. The signal is composed of three components which are the first negative (on-response), the second negative, and the third broad positive components (off-response). Although not shown here, the signal returned gradually to the baseline (also see Figure 6). Results at pH 4.7 are shown in Figure 2, b and b′; the on-response consists of the negative (differential) component and off-response only the positive components different from that at pH 8.8. Hereafter, the differential responses will be displayed to reveal clearly the direction of the potential change.

We conducted experiments using purple membranes. Results obtained are shown in Figure 3. The signals obtained are essentially the same as reported elsewhere,[28,29] although in previous papers purple membranes were dried up to adhere them on the electrode surface while the present method employed

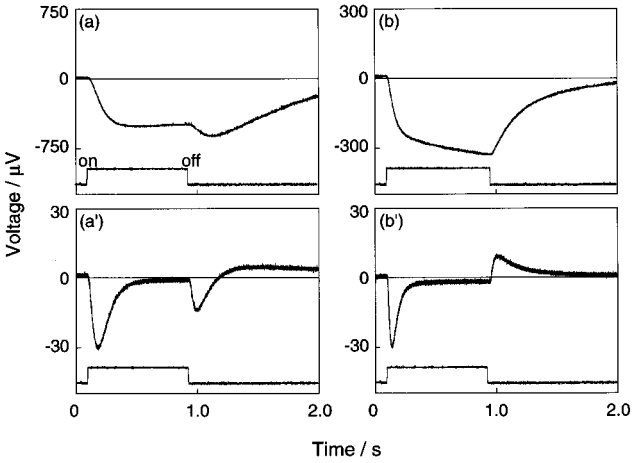

Figure 2. Light-induced photoresponses of *p*pR. The pH was adjusted at 8.8 (a) and 4.7 (b). The upper curve shows the potential change (passed through 0.08 Hz low-cut filter) and lower rectangular signal indicates the period of the illumination with a constant intensity (1.34 kW/m^2). Parts a′ and b′ are the responses obtained by passing through 15 Hz low-cut filter, and are the partially differentiated curves of (a) and (b), respectively. By this process, the direction of the response change becomes clear. The signal of (a′) is composed of three components; at the onset of illumination a negative component was observed, and on removal of light, the negative component again appeared followed by a positive slow component. On the other hand, the signal (b′) is simple: the on-response is composed of a negative component and off-response, a positive component.

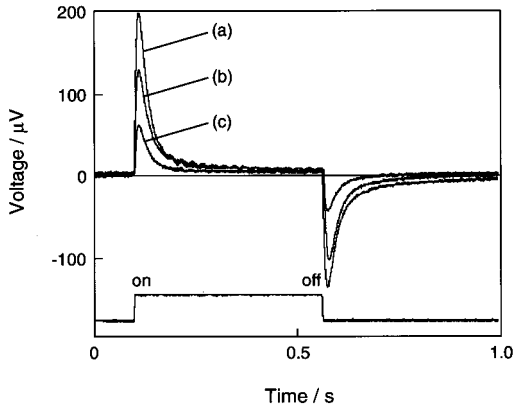

Figure 3. Photoresponses of bR whose concentrations were 150 μM (a), 100 μM (b), and 50 μM (c). Compartment 3 in Figure 1 contained 400 mM NaCl (pH 4.7). This showed that the present method gave essentially the same results as those reported previously[28−30] (for details see text). The positive deflection means the proton release from the protein.

the dialysis membrane. The on-response of bR was positive and off-response negative. Coincidence with previous results[28,29] means that the positive deflection corresponds to the acidification of the solution adjacent to the working SnO_2 electrode (i.e., proton release from the protein) and the negative deflection, the alkalization (proton uptake by the protein). This figure also shows bR-concentration-dependent responses.

Figure 4 is the action spectrum of the first component of the photoresponse. The broken line represents the absorption spectrum of *p*pR; it agrees well with the action spectrum of the response. This proves that the response is originated from *p*pR.

Figure 5 shows the responses observed at alkaline, neutral, and acidic pHs; the off-response (the second and third components) was affected by pH, but the on-response (the first component) remained negative for all pH. We showed in our

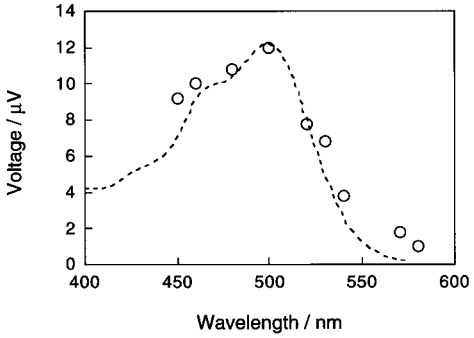

Figure 4. Action spectrum of the first component of the *p*pR photoresponse. The broken line and open circles represent the absorption spectrum of *p*pR and the magnitude of the light-induced transient responses, respectively. The interference filters were used to provide an actinic light of wavelengths (±10 nm).

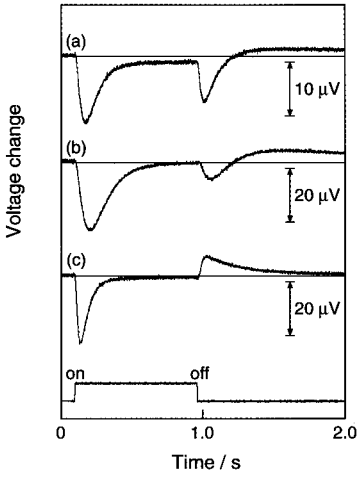

Figure 5. Photoresponse of *p*pR observed at pH 9.8 (a), 7.5 (b), and 3.5 (c). The on-response was negative for all pH, but the pattern of off-response depended on pH which was adjusted by HCl or NaOH.

previous paper[13] that the rate constant of the M decay (O formation), k_1, was pH-dependent while that of O decay, k_2, was relatively pH-independent (see Figure 7 for the definitions of k_1 and k_2). At pH 9.8 k_2 was about 5-times larger than k_1 and at pH 7.5 it was about 3 times larger. At pH 3.5, on the other hand, k_1 was 15 times larger than k_2. At alkaline and neutral pH, M decay was the rate-determining step of the photocycle while at acidic pH, O decay was rate determining. Addition of azide greatly accelerated the M decay (about 300-fold) while azide was less effective in increasing the O decay (5−6-fold) for the entire pH range.[24] Hence, the relation holds for the whole pH range that $k_1 > k_2$ in the presence of azide.[24] At alkaline pH, the response in the presence of azide (50 mM) was similar to that in acidic medium in Figure 5c (data not shown). Therefore, the pH dependence of the off-response may be related to the ratio of the time constants of the M and O decay.

Thus, we did the following experiment: the off-response patterns were examined after the photosteady state was attained. First, *p*pR in the cell was preilluminated for 1 min to reach the photosteady state, and the responses were measured after illumination ceased. Under this condition a single reaction occurred in which intermediates returned to the ground state (M → O → *p*pR). Results obtained at different pH are shown in Figure 6. The ratios of the amounts of the M to O intermediates at the photosteady state were calculated from k_1 and k_2 determined by flash photolysis. From the top trace to the lower, the ratio of the O content increases.

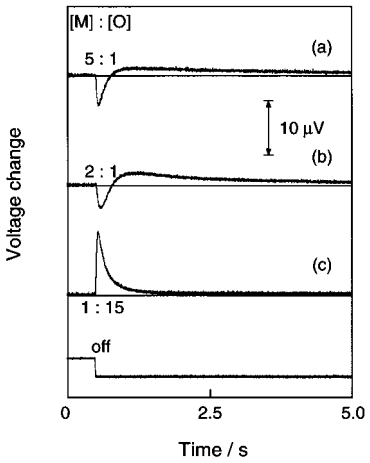

Figure 6. Off-responses from the photosteady state at three pH's of 9.8 (a), 6.8 (b), and 3.9 (c). The content ratios of M and O intermediate at the steady state are changed because of pH dependence of kinetic decay constants of intermediates. The ratio is also shown.

Discussion

As described in the Introduction, SnO_2 electrode is considered to monitor the pH change. If this is correct, the adhesion of the protein to the electrode surface might not be necessary. Therefore, we placed a thin layer of the concentrated protein solution contiguous to the SnO_2 electrode. This is useful for the sample solubilized with detergents such as *p*pR. In previous papers,[25−32,38] the electric current was measured while in the present study the partially differentiated potential change was measured. Comparison of previous data[25−32,38] with Figure 3 reveals that both methods give essentially the same results. The response curves obtained by the present method are somewhat broader, which might come from the difference of the response time of the electric circuit used. Note that a low-cut filter of 15 Hz was used in the present experiment. Thickness of the *p*pR solution layer is 0.5 mm; then the time required for the diffusion of protons released from or uptaken by *p*pR may be another reason of the broad signal, which is especially seen for the last off-response shown in Figures 5 and 6.

First, we should discuss the off-response. The off-response of Figure 6 involves the two processes M → O → *p*pR. As Figure 6 shows, with the increase in the O content in the photosteady state, the positive component increases. This suggests that the decay of the O intermediate coincides with the proton release. The ratio of M to O was calculated from the rate constants determined by the flash photolysis. In the present experiments, however, steady illumination was provided and if the photointermediates are photoactive, the quantitative ratio might be changed. In spite of this, however, the proton release at the O decay is most probable (see discussion below).

Bearing in mind the sequence of the photocycle and the conclusion that the proton release occurs at the O decay, it is very reasonable that the M decay (O formation) coincides with the proton uptake. The half times of the M decay at pH of 9.8, 7.5 and 3.5 are 2.2, 1.4, and 0.03 s, respectively. These are pH values of the response shown in Figure 5. The duration of illumination is 1 s; than under the condition of Figure 5a,b, M is a major photointermediate generated and some decays to the O intermediate. Therefore, the proton upake at the M decay (O formation) followed by the release at the O decay is the most probable in spite of the possibility of the photoreactivity of intermediates as described above. Note that the actinic light never irradiated M. Since the pK_a of Asp-75 (which works as the proton acceptor from the Schiff base on irradiation[19]) is estimated to be about 3 in the present preparation (data not shown, and manuscript in preparation), the partial protonation of Asp-75 may be considered for the response (c), but the flash photolysis reveals the rapid decay of M followed by O formation in this pH.

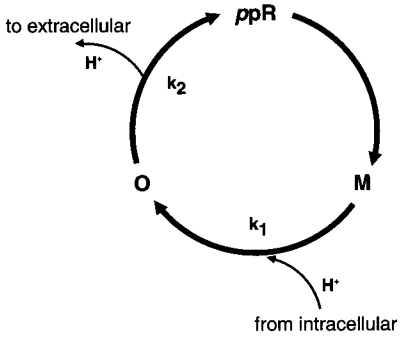

Figure 7. Putative model of the proton uptake and release during the photocycle of *p*pR.

In conclusion, it is shown that the present SnO_2 photoelectrochemical cell is a useful and sensitive method for monitoring the proton transfer of pigment proteins, and this technique may be applicable to other pigment proteins such as rhodopsin, sR, PYP (photoreactive yellow protein), retinochrome, phytochrome, and others. This technique has advantage in that the solubilized membrane protein is applicable because no dry-up process is employed.

Another important conclusion is illustrated in Figure 7 showing our model of the proton-transfer in *p*pR. Engelhard et al.[23] observed the photoelectric current when *p*pR was incorporated into a black bilayer membrane, especially in the presence of azide, which suggests electrogenic proton transport. During formation of the M intermediate, proton is transferred from the Schiff base to Asp-75.[19] In the O intermediate the Schiff base is presumably protonated and Asp-75 is protonated too. Therefore, the Schiff base is reprotonated during the M → O transition; the proton might come from the intracellular space. Because *p*pR lacks residues, analogous to D96 of bR, which donates proton to the deprotonated Schiff base,[39] the M decay is very slow. During O → *p*pR, Asp-75 becomes deprotonated and proton is released to the extracellular space, although this does not necessarily mean that the same proton from Asp-75 is released from the protein. The response pattern of *p*pR resembles that of the bR mutants lacking the proton-releasing complex (Glu-204 and Glu-194).[29,33−36] This raises the idea that *p*pR also lacks the proton-releasing complex. Alignment of the primary amino acid sequence reveals that Glu-204 of bR corresponds with Asp-193 of *p*pR and Glu-194 of bR, Pro-183 of *p*pR. Hence the measurement of the photoresponse of P183E *p*pR mutant is of interest and is now in progress in our laboratory.

Sasaki and Spudich[40] also showed the similar proton uptake and release of sRII from *H. Salinarum*, but they described that proton is not transported, which is contrary to *p*pR. The molecular basis which can account for this difference is also interesting and merits further investigation. The question of how the proton transfer of *p*pR shown in the present paper functions in the signal transmission to Htr-II awaits further investigation. It is noted that sR (or sR-I) also pumps proton when sR is free from Htr.[41,42]

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