Low-temperature photoreaction cycle of phoborhodopsin (sensory rhodopsin II) from Halobacterium salinarium

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Low-temperature photoreaction cycle of phoborhodopsin (sensory rhodopsin II) from *Halobacterium salinarium*

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There are four types of retinal proteins in the membrane of *Halobacterium salinarum.* Bacteriorhodopsin (BR) and halorhodopsin (HR) convert light energy into electrostatic gradients that can be used by the cell as an energy source to produce ATP. Sensory rhodopsin I (SR I) and phoborhodopsin (pR) enable the cells to migrate toward an environment optimal for light energy harvesting while avoiding potentially damaging shorter wavelength light. In the present work, low temperature photoreaction cycle of pR expressed in *E. coli* was studied. Comparing to the previous results, the new findings in the present work are: (i) The K-like intermediate was found to be a mixture of two photoproducts. (ii) Formation of an L-like intermediate (P482) was observed. (iii) Upon light irradiation, formation of a long lived shorter wavelength photoproduct (P370) was observed at 20℃.

Keywords: Phoborhodopsin, Sensory rhodopsin II, Photointermediate

1 INTRODUCTION

There are four types of photoactive retinal proteins in the membrane of *Halobacterium salinarum*: bacteriorhodopsin and halorhodopsin are light-driven ion pumps transporting protons or chloride ions across the membrane, respectively; sensory rhodopsin I and phoborhodopsin are photo receptors responsible for the phototaxis of the bacterium. Among the four retinal proteins, BR has been most extensively studied. Light-activation causes the protein undergo a photoreaction cycle with a series of intermediates designated as K, L, M, N and O, during one photocycle a proton is transported across the membrane[(1)]. pR was first reported in 1985 as the fourth retinal pigment for the photo-repellent response of the bacterium[(2)]. Although pR is a retinal protein with seven helical structures like BR, there are many differences between BR and pR: pR works as a photo-sensor, while BR works as an ion pump; the absorption maximum of pR is more than 80 nm shorter than that of BR; the time required for the completion of the pR photocycle is much longer than that of BR. It would be worthwhile to study the molecular origin of these differences. However pR is unstable under low ionic strength conditions and the content in the cell membrane is much lower than the other retinal proteins that made its purification difficult. Thus the publications of the study on pR are not so many.

Fortunately, an expression system of pR (designated as *s*pR hereafter) in *Escherichia coli* (*E. coli*) and purification methods with a Ni-column were developed recently[(3)], which enables the detailed studies on the photochemical properties of *s*pR. In the present work, the low-temperature photoreaction of *s*pR was studied.

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Based on our results a photocycle was proposed and compared with previously published one[4].

2 METHODS

Construction of expression vector and preparation of proteins. The sopII gene was amplified by PCR from the extracted genome DNA from *Halobacterium salinarum*. For PCR, the 5'-primer with a restriction site *Nde* I (5'-CATATGGCACTCACGACATGGTTTTG-3') and the 3'-primer with a restriction site *Xho* I (5'-CTCGAGGTCGCCGTCCGGCGTTGGTTC-3') were designed. Underlined bases indicate the introduced restriction site. The PCR products obtained were purified and subcloned into the vector pGEM-T Easy (Promega). The *Nde* I – *Xho* I fragment from this plasmid were cloned in corresponding sites of the vector pET21c (Novagen), and the *s*pR-expression plasmid (pET21c/*s*pRHis) was constructed. The sequences of the PCR products were confirmed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

The expression and preparation of proteins were essentially the same as those described previously by Mironova *et al*[3]. *E.coli* BL21-CodonPlus (DE3)-RP cells (Stratagene) were transformed by pET21c/*s*pRHis. The cells were grown in 2× YT medium supplemented by ampicillin (final concentration of 50 μg/ml) to OD_{660} of 0.3–0.4 at 37 °C. Induction was initiated by addition of 1 mM isopropyl-1-thio-β-galactoside (IPTG) and 10 μM all-*trans* retinal (Sigma-Aldrich). After another 3 h of cultivation, the cells were harvested by centrifugation (4,700×*g*, 10 min, 4 °C) and suspended in Buffer A (500 mM NaCl, 1 mM EDTA and 50 mM MES, pH 6.0). The cells were washed by centrifugation (3,300×*g*, 10 min, 4 °C) and resuspended in Buffer A, followed by sonication on ice. The cell debris were removed by centrifugation (3,300×*g*, 3 min, 4 °C) and membrane fraction was collected by ultra-centrifugation (106,000×*g*, 1.5 h, 4 °C). The membrane was suspended in Buffer S (4 M NaCl, 50 mM MES, pH 6.0) and solubilized by 1% n-dodecyl-β-D-maltoside (DDM) under stirring overnight at 4 °C in the dark. The unsolubilized materials were removed by ultra-centrifugation (106,000×*g*, 1.0 h, 4 °C). The supernatant was applied to a Ni-NTA agarose (Qiagen) column equilibrated with Buffer S. The run of this column was carried out in the dark to prevent the bleach of the pigment by light. The column was washed thoroughly with Buffer W (4 M NaCl, 20 mM imidazole, 50 mM MES, pH 6.0 and 0.05 % DDM) and non-specific binding materials were removed. Protein (*s*pR) was eluted by Buffer E (4 M NaCl, 250 mM imidazole, 50 mM MES, pH 6.0 and 0.05 % DDM). Imidazole was, finally, removed by Sephadex (G-25, GE healthcare) and the sample was concentrated by ultrafiltration (Amicon Ultra-15, Millipore). During the preparation of the pigment, we tried not to expose the sample under light as much as possible.

Low-temperature spectrophotometry. For measuring the absorption spectra at low temperature, the purified *s*pR in 4 M NaCl, 10 mM 7mix Buffer (mixture of citrate, MES, HEPES, MOPS, CHES, Tris, CAPS, the final concentration of each was 10mM), pH 5.0 and 0.05 % DDM was suspended with glycerol in a final concentration of 66 %. Absorption spectra were measured by MPS 2000 recording spectrophotometer (Shimadzu) equipped with a glass cryostat as described previously[5]. The temperature of the sample was monitored with a copper-constantan thermocouple attached to the cell holder. The sample was irradiated with light from 300 W halogen projector lamp passed through an interference filter and/or cut-off filter. In order to reduce heat of the light, the irradiation light was passed through a 5 cm water layer.

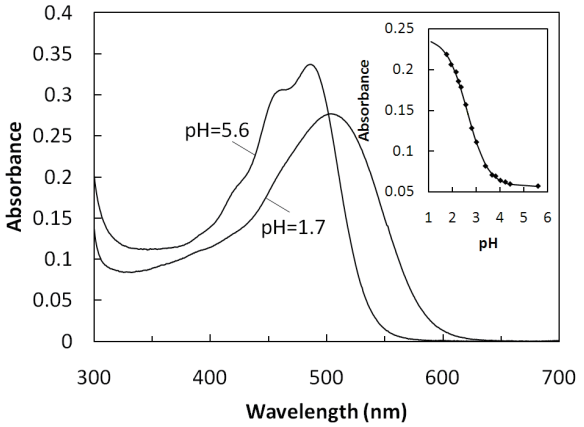

Fig. 1 Absorption spectra of *s*pR at pH 5.6 and 1.7 at 20 °C. Inset shows the pH titration curve at 535 nm. spR was suspended in 200 mM NaCl, 10 mM 7 mix Buffer.

3 RESULTS

3.1 pH titration of *s*pR

Acidification of BR causes spectral red shift due to the protonation of Asp85 which is the counter ion of the protonated Schiff base. The pKa value of Asp85 of BR is 2.6[6]. The corresponding amino acid in *s*pR is Asp73. To determine the pKa of Asp73, we measured the absorption spectrum of *s*pR under different pH conditions from 5.6 to 1.7. The absorption spectra of *s*pR at pH 5.6 and 1.7 and the titration curve are shown in Fig. 1. The absorption maximum of *s*pR at pH 5.6 was at 486 nm. It became 503 nm at pH 1.7 with a decrease in the molar extinction coefficient. It is noteworthy that the vibrational fine structure became less prominent at pH 1.7. The calculated pKa of Asp73

was 2.6±0.02 that is the same with that of BR (2.6) and smaller than that of *phraonis* phoborhodopsin (3.5) [7], a homolog of *s*pR found in *Natronomonas pharaonis*.

3.2 Photoreactions of *s*pR at -180 °C

The purified *s*pR in the presence of 0.05 % DDM and 66 % glycerol showed its absorption maximum at 486 nm with three shoulders at 452, 419 and 392 nm at -180 °C (curve 1 in Fig. 2). When irradiated with 450 nm light, formation of K-like intermediate, P500, was observed as previously reported [4,8]. A photo-steady state (PSS_{450}, curve 2 in Fig. 2) was obtained by prolonged irradiation with 450 nm light.

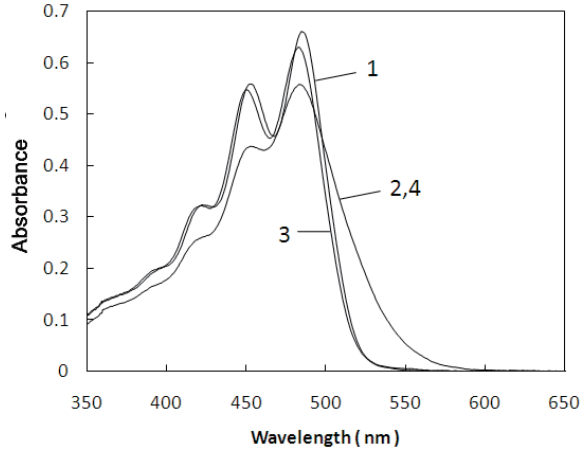

Fig. 2 Photoreaction of spR
Curve 1, spR. Curve 2, photo-steady state (PSS_{450}) obtained by irradiating spR (curve 1) with 450 nm light for 64 min. Curve 3, Photo-steady state (PSS_{540}) obtained by irradiating PSS_{450} (curve 2) with >540 nm light for 32 min. Curve 4, Photo-steady state (PSS_{450}) obtained by irradiating PSS_{540} (curve 3) with 450 nm light for 64 min.

Subsequently, PSS_{450} was irradiated with >540 nm light which was supposed to be absorbed mainly by P500. The transition of the longer wavelength photoproduct into a shorter wavelength photoproduct was observed. The transition seems to be a reverse reaction of P500 into original spR. However, absorption spectrum of the photo-steady state mixture (PSS_{540}, curve 3 in Fig. 2) obtained by prolonged irradiation with >540 nm light did not overlap with that of spR. The maximum absorbance of PSS_{540} was at 483 nm that is slightly blue-shifted from that of *s*pR, and the extinction coefficient seems to be slightly small, suggesting the formation of a shorter wavelength photoproduct. The estimated absorption maximum of this photoproduct was at 481 nm (data was not shown), thus, it should be the shorter wavelength photoproduct, P480, described by Imamoto *et al*[4].

Irradiation of PSS_{540} (curve 3 in Fig. 2) by 450 nm light gave a photo-steady state (curve 4 in Fig. 2) that was identical to PSS_{450} (curve 2 in Fig. 2) produced by irradiation of *s*pR with 450 nm light. Thus, PSS_{450} (consisting of P500, P480 and *s*pR; curve 2 and 4 in Fig. 2) and PSS_{540} (consisting of P480 and *s*pR; curve 3 in Fig. 2) are perfectly photo-reversible.

Closer inspection of the transition from PSS_{450} to PSS_{540} reveals that it was not as simple as P500 converted to spR or P480, it was appeared to be a multiphase transition. The difference spectrum of early transition and that of later one were calculated and normalized (Fig. 3). The normalized difference spectra showed that they are different especially in the longer wavelength region.

We concluded that there exist at least two longer wavelength photoproducts named P500 and P500'.

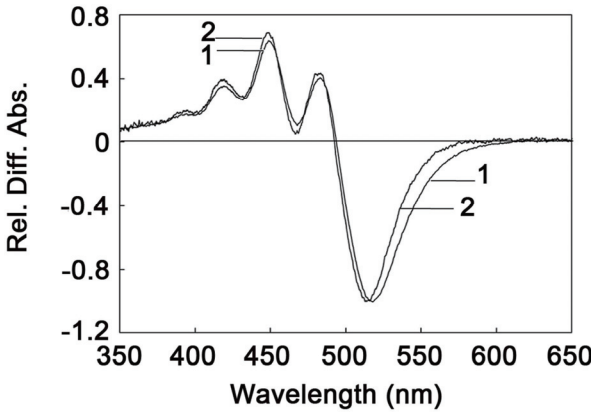

Fig. 3 Comparison of the difference spectra
Curve 1, normalized difference spectra between the photoproduct mixture obtained by irradiating PSS_{450} with >540 nm light for 1min and spR. Curve 2, normalized difference spectra between PSS_{540} and the photoproduct mixture obtained by irradiating PSS_{450} with >540 nm light for 4min. Curve 1 represents the early transition and Curve 2 represents the late transition.

3.3 Thermal reaction of P480

PSS_{540}, mixture of P480 and *s*pR, was warmed to the pre-determined temperature (-160, -140, -130 and -120 °C) and immediately cooled down to -180°C to measure the spectra. The absorption spectrum of the mixture gradually shifted toward that of spR with the temperature increase as reported in the previous paper[6] indicating that P480 was thermally converted to *s*pR by the warming above -160 °C (data were not shown).

3.4 Thermal reaction of P500 and P500'

PSS_{450} was warmed up and spectral changes by a stepwise warming of 10 °C were recorded. Spectral changes within-140 to -100 °C appeared to be small and noisy (data were not shown).

We could not measure the spectra in the temperature range between -100 °C and -80 °C, because of the baseline change due to glycerol melting in the cuvette. The spectra of thermal products above -80 °C were measured at -80 °C. By warming to -60°C the absorbance decrease in the longer wavelength region

was observed (curve 1 in Fig. 4a). By further warming up to -40 °C, absorbance decrease in the longer wavelength region and a concomitant increase at 350 nm were observed as shown in curve 2 and 3 in Fig. 4a indicating the formation of M-like intermediate, P360 as previously reported[8]. It is noteworthy that the shape of the negative bands of curve 1 is quite different from those of curve 2 and 3. One of possibilities causing this difference is the formation of a new intermediate corresponding to L-intermediate of BR. The estimated difference maximum of this photoproduct is at 482 nm, thus we named it P482.

No obvious spectral change was observed by warming to -30 °C or -20 °C (curve 4 and 5 in Fig. 4a). By warming to above -10 °C, the formation of O-like intermediate, P540 and a concomitant recovery of *s*pR were observed (curve 1 and 2 in Fig. 4b). The recovery to *s*pR completed at 10 °C (curve 3 in Fig. 4b).

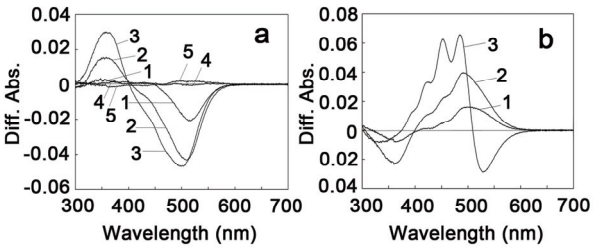

Fig. 4 Thermal reaction of PSS_{450} from -80 to 10 °C
Panel a: Curve 1-5 indicate the difference spectral changes before and after each stepwise warming of PSS_{450} from -80 °C to -60, 50, -40, -30, -20 °C, respectively. Panel b: Curve 1-3 indicate the difference spectral changes before and after each stepwise warming of PSS_{450} from -80 °C to -10, 0, 10 °C, respectively. Each time immediately after warming to predetermined temperature, the mixture was cooled down to -80 °C to measure the absorption spectra.

3.5 Formation of P370 by irradiation of *s*pR at 20 °C

Longer irradiation of *s*pR with 450 nm light at 20 °C caused formation of a new photoproduct with a difference maximum at 370 nm (Fig. 5). This photoproduct was tentatively named P370. It slowly reverted to the original *s*pR in the dark at 20 °C with a half time of 190 min (data not shown).

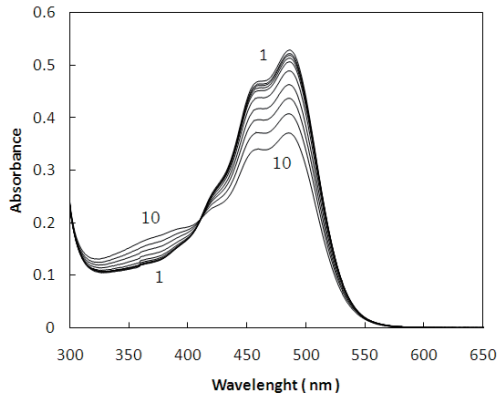

Fig. 5 Photoreaction of spR at 20 °C
*s*pR (at 20 °C; curve 1) was irradiated with 450 nm light for a total period of 1, 2, 4, 8, 16, 32, 64, 128 and 256 min (curve 2 – 10, respectively).

4. CONCLUSION

The photoreaction cycle was concluded in Fig. 6. Comparing to the previous results, new findings in the present work are listed as follows: (i) The k-like intermediate (P500) was a mixture of two photoproducts. (ii) Formation of an L-like intermediate (P482) was observed on the conversion process from K-like intermediates (P500 and P500`) to M-like intermediate (P360). (iii) On long irradiation of *s*pR at 20 °C, formation of a new photoproduct (P370) was observed and it decayed to the original *s*pR in the dark with a decay half time of 190 min. The N-like intermediate was not detected under our experimental conditions.

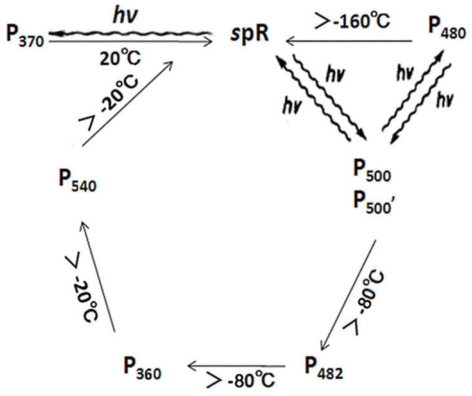

Fig. 6 Photocycle of pR
Wavy lines represent the photoreactions, straight lines represent the thermal reactions. P_x represents the photo-intermediates, x indicates the difference absorbance maxima of them.

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