Photocycle of Phoborhodopsin from Haloalkaliphilic Bacterium (*Natronobacterium pharaonis*) Studied by Low-Temperature Spectrophotometry†

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ABSTRACT: Phoborhodopsin (pR) is the fourth retinal pigment of *Halobacterium halobium* and works as a photoreceptor for the negative phototactic response. A similar pigment was previously found in haloalkaliphilic bacterium (*Natronbacterium pharaonis*) and also works as the receptor of the negative phototactic response; this pigment is called *pharaonis* phoborhodopsin (ppR). In this paper, the photocycle of ppR was investigated by means of low-temperature spectrophotometry. The absorption maximum of ppR is located at 498 nm, while that of pR is at 487 nm. The absorption spectra of the two have similar vibrational structures. Irradiation of ppR below −100 °C produced a K-like intermediate (ppR_K) which was a composite of two components. The original ppR and ppR_K were perfectly photoreversible. On warming, ppR_K was directly converted to an M-like intermediate without formation of the L-like intermediate. The M-like intermediate was converted to the O-like intermediate at pH 7.2, but the O-like intermediate was not detected at pH 9.0. The O-like intermediate then reverted to the original pigment. On the basis of these findings, the photocycle and the primary photochemical process of ppR are presented.

Halobacterium halobium has the fourth retinal pigment found following bacteriorhodopsin (bR),[1] halorhodopsin (hR), and sensory rhodopsin (sR) (Takahashi et al., 1985; Tomioka et al., 1986a; Wolff et al., 1986). This fourth pigment (absorption maximum at 487 nm) is a photoreceptor for negative phototaxis and is called phoborhodopsin (pR). The photochemical reaction of pR in the cell membrane has been investigated by flash photolysis on a millisecond time scale at room temperature, and two intermediates were found; they had difference absorption maxima at 350 (P350) and 530 nm (P530), respectively. These intermediates may correspond to M and O intermediates of bR, respectively (Tomioka et al., 1986a). Furthermore, the presence of another intermediate (P520), which may correspond to K intermediate of bR, was shown by low-temperature spectrophotometry (Imamoto et al., 1991; Shichida et al., 1988). The use of a purified sample would be indispensable for obtaining more detailed information on the photocycle of pR; however, the purification of pR is difficult because all detergents examined so far bleached it, so that column chromatographic techniques cannot be applied.

It was recently reported that a haloalkaliphilic bacterium (*Natronobacterium pharaonis*) had retinal pigments (Bivin & Stoeckenius, 1986). The absorption spectrum of one of the pigments (λ_{max} at about 500 nm) is very similar in shape to that of pR, except for a 10-nm red-shift. This pigment also has a photoreaction cycle similar to that of pR; at least two intermediates (difference absorption maxima, 390 and 560 nm) are identified which correspond to P350 and P530 of pR, respectively. We also confirmed the presence of these intermediates (Tomioka et al., 1990). In addition, it was shown that this pR-like pigment in *N. pharaonis* worked as a photoreceptor for the negative phototactic response of this microorganism (Tomioka et al., 1990). Thus, we call this pigment *pharaonis* phoborhodopsin (ppR). ppR may be a good material for investigating the molecular mechanism of negative phototaxis of the bacterium because, unlike pR, it was stably solubilized with several kinds of detergents and purified by column chromatography.

The present paper describes analysis of the photochemical and subsequent thermal reactions of ppR in a partially purified sample by low-temperature spectroscopy. Current findings clearly indicate that ppR has a photoreaction cycle similar to that of pR with a K-like intermediate but not an L-like intermediate. Furthermore, detailed analysis indicated that the K-like intermediate is a mixture of two components having different spectral shapes and thermal stabilities.

Materials and Methods

Strain and Medium Conditions. N. pharaonis (NCMB 2191) was obtained from the National Collection of Industrial and Marine Bacteria, Ltd. (Scotland). The culture medium was composed of the following reagents (w/v): 1.0% yeast extract (Difco or Kyokuto), 0.75% casamino acid (Difco or Kyokuto), 0.3% trisodium citrate, 0.2% KCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.000036% $MnCl_2 \cdot 4H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 20.0% NaCl, and 5.0% $Na_2CO_3 \cdot 10H_2O$, pH 9.5. Sodium carbonate and the other reagents were autoclaved separately and then mixed together after cooling to room temperature. No precipitation was observed.

Membrane Preparation. N. pharaonis was grown aerobically in 20 L of the culture medium at 45 °C for 3 days, and the cells in the stationary phase were harvested. After washing

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[1] Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; pR, phoborhodopsin, λ_{max}, absorption maximum in the visible region; ppR, *pharaonis* phoborhodopsin.

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with 4 M NaCl by centrifugation (10500*g*, 20 min), the cells were resuspended in 400 mL of 4 M NaCl. The suspension was completely frozen by immersing it in liquid nitrogen and then thawed. This freeze–thaw procedure was repeated again. Then the volume of the suspension was adjusted to 840 mL with 4 M NaCl, and 10 mg of DNase 1 (Sigma) was added, followed by stirring for a few hours at room temperature. After removal of any large debris by centrifugation at 10500*g* for 20 min, the supernatant was centrifuged at 186600*g* for 60 min. The pellet was washed twice with 4 M NaCl and then resuspended in 4 M NaCl.

Purification of ppR. Earlier papers (Ogurusu et al., 1984; Duschl et al., 1988, 1990) were suggestive in the purification of ppR. All steps described below were carried out at room temperature. The membrane suspension (210 mL) was mixed with 70 mL of 20% sodium cholate and stirred for 30 min. Insoluble materials were removed by centrifugation (186600*g*, 60 min) at 15 °C. The supernatant was applied to a column of phenyl-Sepharose CL-4B (Pharmacia, 2.6 × 5.0 cm) which had been equilibrated with buffer A (2 M NaCl, 25 mM Tris-HCl, 0.4% sodium cholate, pH 7.2). The column was washed with 300 mL of buffer A. ppR was then eluted from the column with buffer B (2 M NaCl, 25 mM Tris-HCl, 0.5% octyl glucoside, pH 7.2). Fractions containing ppR were put into a dialysis tube, followed by placing the tube in poly(ethylene glycol) to be concentrated. The concentrated fraction then applied to hydroxyapatite column (HCA-100S, Mitsui Toatsu Chemicals, Inc.), which had been equilibrated with buffer B. ppR was eluted with buffer B by natural flow. Orange fractions containing ppR were collected and then mixed with the same volume of buffer C (25 mM Tris-HCl, 3.0% sodium cholate, pH 7.2). Solid ammonium sulfate was then added to the mixture to 20% saturation. After stirring for 30 min, it was applied to a column of octyl-Sepharose CL-4B (Pharmacia, 1.5 × 5.0 cm), which had been equilibrated with buffer D (1 M NaCl, 20% saturated ammonium sulfate, 1.5% sodium cholate, 25 mM Tris-HCl, pH 7.2). ppR was eluted with buffer E (1 M NaCl, 20% saturated ammonium sulfate, 0.06% sodium cholate, 0.3% octyl glucoside, 25 mM Tris-HCl, pH 7.2). The orange fractions were dialyzed against buffer F (4 M NaCl, 25 mM Tris-HCl, 0.5% octyl glucoside, pH 7.2) overnight, followed by concentration by means of ultrafiltration (Amicon, membrane YM-30), and this concentrated solution was used as a sample for optical experiments. The absorption spectrum of the sample at room temperature is shown in Figure 1. This curve has a maximum at 498 nm and a shoulder at about 470 nm, although the absorption spectrum of heme protein(s) (λ_{max} at about 420 nm), which was contaminated in the sample, was superimposed. SDS–PAGE is also shown in Figure 1, illustrating that ppR is main component and that the molecular weight of ppR is 2.4×10^4. When further purification was required, the third column chromatography (octyl-Sepharose chromatography) was repeated.

Low-Temperature Spectrophotometry. To prepare the sample of pH 9.0, the sample was dialyzed against buffer F, the pH of which was adjusted to 9.0. Glycerol was then mixed in at a final concentration of 66% (ppR sample). For spectral measurements at low temperature, an MPS-2000 recording spectrophotometer (Shimadzu) equipped with a special glass cryostat was used (Yoshizawa & Shichida, 1982). The temperature of the sample was monitored with a copper–constantan thermocouple attached to a sample cell holder. The sample was irradiated with a light from a 1-kW tungsten–halogen lamp (Sanko) which had passed through an interference filter (Nihonshinku) or a glass cut-off filter (Toshiba). Heat radiation in the light was removed by passing it through a 5-cm water layer. For correction of the light scattering of the sample, opal glass was placed in both of the sample and reference beams.

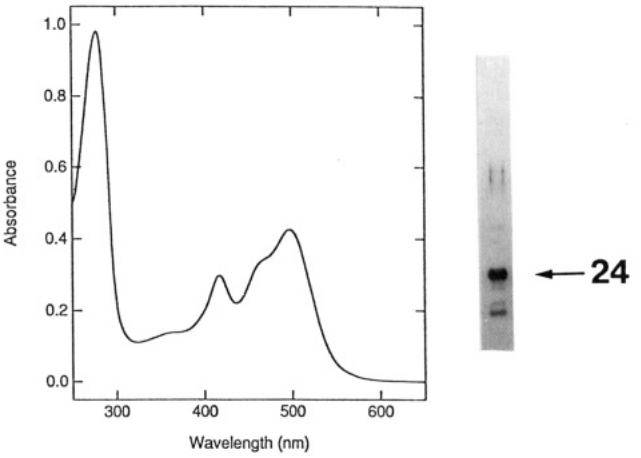


FIGURE 1: Absorption spectrum and SDS–polyacrylamide gel electrophoresis of the partially purified ppR. Gel electrophoresis was done by the method of Laemmli (1970), and bands were visualized by the silver stain. The position of the ppR band is shown by the arrow with the estimated molecular mass (in kilodaltons).

RESULTS

The flash photolytic experiment of ppR at room temperature revealed the formation of two intermediates at pH 7.2, whose difference absorption maxima were located at 390 and 560 nm, respectively (Bivin & Stoeckenius, 1986; Tomioka et al., 1990). They are tentatively designated as ppR_M and ppR_O here because their spectroscopic and thermal properties seem to correspond to M and O intermediate of bR, respectively. Our preliminary experiments by flash photolysis at room temperature showed that the interpretation of data on the photocycle at pH 7.2 was complicated, because the time constants of formation and decay of ppR_M and ppR_O were close to each other. After examination of the experimental conditions, we found that ppR_O was hardly detected under an alkaline condition (pH 9.0), as is true for the O intermediate of bR. We therefore investigated the photochemical reactions of ppR at low temperatures at pH 9.0.

First, the absorption spectrum of ppR sample was recorded at −100 °C (Figure 2a, curve 1). On irradiation with a blue light (436 nm) at −100 °C, a remarkable increase of absorbance at about 540 nm with a concurrent decrease below 500 nm was observed (Figure 2a, inset). These spectral changes indicate the formation of a photoproduct which corresponds to the K intermediate of bR (designated as ppR_K). Prolonged irradiation formed a photo-steady-state mixture composed of ppR_K and the original ppR (Figure 2a, curve 8). Subsequent irradiation with light (>580 nm) converted the photoproduct back to the original ppR (Figure 2b, curve 16). Reirradiation of this sample with 436-nm light produced the same photo-steady-state matrix (Figure 2c, curve 17), indicating that the original ppR and ppR_K were perfectly photoreversible at −100 °C. On irradiation at liquid-nitrogen temperature, the difference spectra were almost identical to those between the original ppR and ppR_K and did not change on warming to −100 °C (data not shown), indicating that the same photoreaction took place at liquid-nitrogen temperature as at −100 °C.

In the photoreversion process of ppR_K to the original ppR irradiated with >580-nm light, the relative absorption change

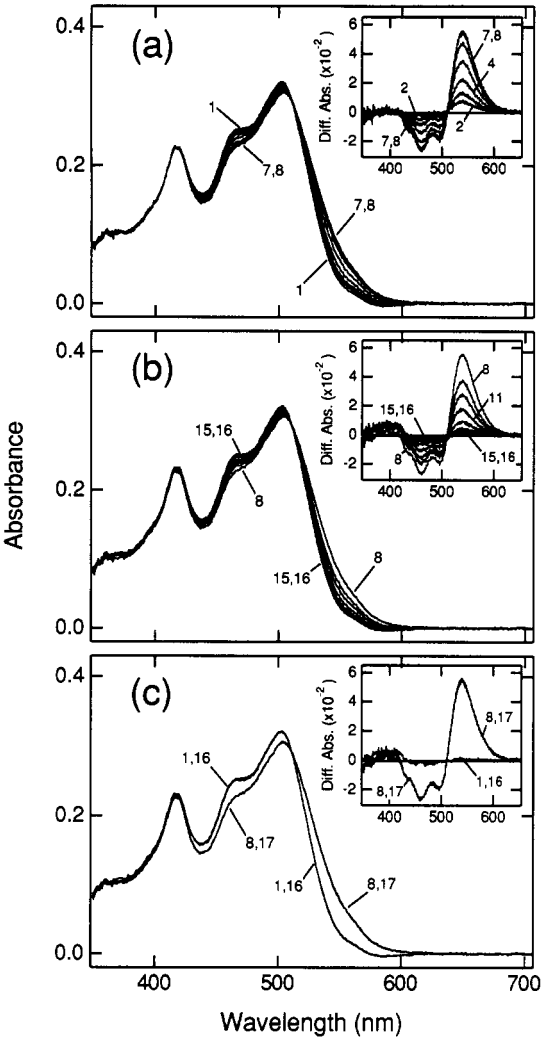


FIGURE 2: Formation of ppR_K (K-like intermediate) and interconversion between the original ppR and ppR_K by irradiation at −100 °C. (a) Spectral changes in the course of conversion of the original ppR to ppR_K. The ppR sample (66% glycerol, pH 9.0) (curve 1) was irradiated with 436-nm light at −100 °C for a total of 5, 10, 20, 40, 80, 160, and 320 s (curves 2–8, respectively). Curve 8 is a photo-steady-state mixture composed of ppR and ppR_K. The inset shows the change of the difference spectrum when the absorption spectrum of the original sample (curve 1 in the main panel) was used as the baseline. (b) Spectral changes in the course of conversion of ppR_K to ppR. The photo-steady-state mixture (curve 8) was irradiated with >580-nm light at −100 °C for a total of 5, 10, 20, 40, 80, 160, 320, and 640 s (curves 9–16, respectively). The inset shows the change of the difference spectrum when curve 1 was used as the baseline. (c) Spectral changes of interconversion between the original ppR and ppR_K. Curve 1 is the ppR sample (identical with curve 1 in panel a). Curve 8 is the photo-steady-state mixture composed of ppR and ppR_K (identical to curve 8 in panels a and b). Curve 16 is a product formed by irradiation of the photo-steady-state mixture with >580-nm light (identical to curve 16 in panel b). Curve 16 is perfectly coincident with the spectrum of the original ppR sample (curve 1). Curve 17 represents the spectrum of a photo-steady-state mixture produced by reirradiation of the product (curve 16) with 436-nm light for 320 s. Curve 17 is perfectly coincident with curve 8. These results indicate the photoreversibility between ppR and ppR_K at −100 °C.

at 540 nm was plotted against the exposure time on the semilogarithmic scale. We found that the kinetic trace was not expressed by a single exponential (data not shown), an indication that the photoreversion was composed of at least two processes. To ensure that it was absorbable only by ppR_K, the irradiation light was changed to >630-nm light and the kinetics of the photoreversion process of ppR_K to ppR were then recorded (Figure 3b). The results revealed that ppR_K

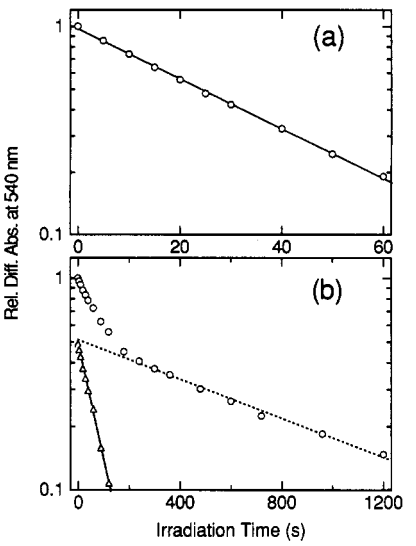


FIGURE 3: Kinetics of photoconversion from ppR to ppR_K (a) and of the photoreversion of ppR_K to the original ppR (b). The relative absorption changes at 540 nm were plotted against the exposure time on a semilogarithmic scale on irradiation of the ppR sample with 436-nm light (a) or on irradiation of ppR_K with >630-nm light (b) at −100 °C. In panel b, the slow component (dotted line) was subtracted from the kinetic curve, and the absorption changes of the fast component were replotted (triangles). This confirms that ppR_K is composed of two photoproducts.

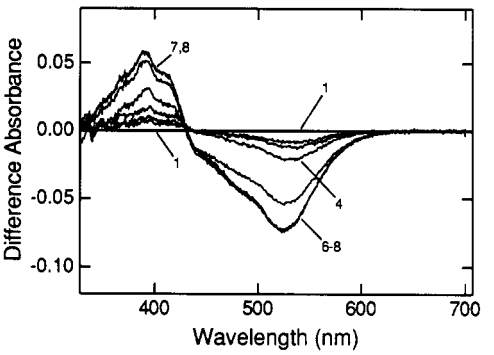


FIGURE 4: Difference spectral changes in the course of warming of the photo-steady-state mixture containing the original ppR and ppR_K from −100 to −30 °C. The photo-steady-state mixture was produced by irradiation of the ppR sample at −100 °C for 320 s with 436-nm light. The absorption spectrum of the mixture at −100 °C was used as the baseline (curve 1). Then the mixture was successively warmed to −30 °C at intervals of 10 °C. Each time, it was recooled to −100 °C, and the spectrum was recorded (curves 2–8).

was composed not of a single component but of two components, $ppR_K(L)$ and $ppR_K(S)$, the spectrum of the former being at a longer wavelength than that of the latter (for details see below). The original ppR, it is noted, is a single component because the kinetics of the conversion process of the original ppR to ppR_K was expressed by a single exponential (Figure 3a).

Figure 4 shows the thermal decay of ppR_K. The difference absorption spectrum of the photo-steady-state mixture before and after warming to one of the required temperatures in the range from −100 to −30 °C was obtained: the sample was warmed to the predetermined temperature, the temperature then reduced suddenly to −100 °C, and the spectrum was recorded at this temperature. With the warming, absorbance at about 535 nm decreased with a concurrent increase at about 390 nm; this spectral change appeared to indicate the conversion of ppR_K to ppR_M. The negative peak, however, was shifted from 535 to 525 nm as the temperature rose, implying either a difference in thermal stability between the two kinds

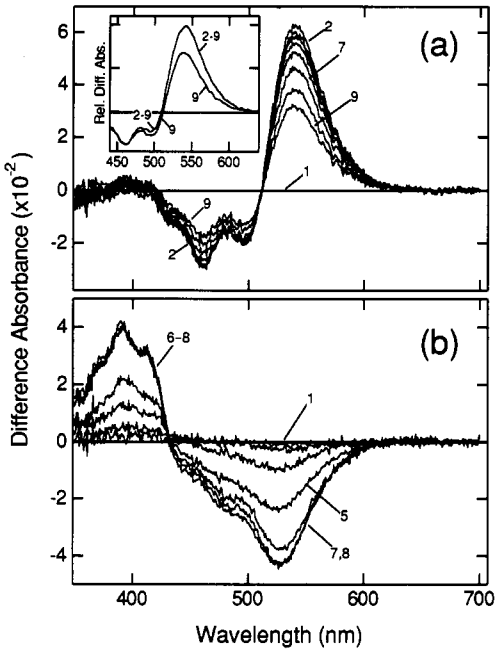


FIGURE 5: Formation of $ppR_K(S)$ intermediate and its thermal reactions. (a) The ppR sample (curve 1, baseline) was irradiated with 436-nm light for formation of a photo-steady-state mixture of ppR_K and the original ppR (curve 2). This mixture was irradiated with >630-nm light for a total of 5, 10, 20, 40, 80, 160, and 240 s at −100 °C (curves 3–9, respectively) to convert the $ppR_K(L)$ intermediate to the original ppR. The inset shows the comparison of curve 9 with the difference spectrum between curves 2 and 9. These two curves were normalized at 460 nm. (b) The difference spectral changes of $ppR_K(S)$ intermediate when warmed from −100 to −30 °C. The sample containing $ppR_K(S)$ intermediate (curve 9 in Figure 5a) was used as a baseline (curve 1). The sample was then warmed up to −30 °C at 10 °C intervals. Each time, it was recooled to −100 °C, and the spectrum was recorded (curves 2–8).

of ppR_K [$ppR_K(L)$ and $ppR_K(S)$] or the formation of an intermediate corresponding to the L intermediate (ppR_L) in the conversion process of ppR_K to ppR_M.

To determine which hypothesis was correct, we carried out the following experiment. Since both of the components of ppR_K [$ppR_K(L)$ and $ppR_K(S)$] were stable below −100 °C, it was unlikely that a putative ppR_L was formed below −100 °C. Figure 3b shows that irradiation with >630-nm light for 240 s converts the component of ppr_K which had absorption maximum at the longer wavelength [$ppR_K(L)$] to the original ppR almost perfectly. Figure 5a (main panel) shows the spectrum changes during this irradiation. Curve 2 shows the spectrum of the mixture of $ppR_K(L)$ and $ppR_K(S)$, and curve 9 shows that obtained after irradiation of the mixture with >630-nm light for 240 s to convert $ppR_K(L)$ to the original ppR. The inset of Figure 5a shows the comparison of curve 9 [the difference spectrum of $ppR_K(S)$ relative to the original ppR] with the difference spectrum between curves 2 and 9 [the difference spectrum of $ppR_K(L)$]. The λ_{max} of positive peaks is clearly shifted.

After irradiation with >630-nm light for 240 s at −100 °C, the rest of ppR_K [$ppR_K(S)$] was warmed. Difference absorption changes before and after warming are shown in Figure 5b, where the wavelength of the negative peak (525 nm) did not shift with the rise in temperature. These results demonstrated that the shift of the negative peak observed in Figure 4 was due to the parallel conversion of two kinds of ppR_K to ppR_M.

Another important suggestion is shown in Figure 5b. If ppR_L is present in the photocycle of ppR, the negative peak must shift to shorter wavelength, because the absorption maximum of ppR_L is assumed to be adjacent to that of the original ppR, as in the cases of rhodopsin and other retinal pigments of *H. halobium* (bR, hR, or sR). Figure 5b shows no shift, however, showing that there is no formation of the intermediate in the process of thermal conversion of $ppR_K(S)$ to ppR_M. The possibility that an L-like intermediate is produced from $ppR_K(L)$ is also ruled out for the following reason. If the putative L-like intermediate with a spectrum similar to that of ppR is present, the wavelength of the negative peaks at the later stage of the thermal conversion of ppR_K (Figure 4) should be shorter than those of $ppR_K(S)$ (Figure 5b). The results contradict this supposition; therefore, it is unlikely that ppR_L is present.

The experiments described above were performed under an alkaline condition. Now the question arises whether ppR lacks ppR_L in the photocycle only under this condition. To clarify this, a ppR sample adjusted to pH 7.2 was used for the low-temperature experiments (Figure 6). As described earlier, the thermal reactions of the intermediates in the sample at pH 7.2 were troublesome to analyze because of the formation of ppR_O. The experiment was carried out as follows.

As a baseline, the absorption spectrum of the ppR sample at pH 7.2 was recorded at −100 °C (Figure 6a). After warming the sample to −80 °C, it was irradiated with 436 nm light to produce not only ppR_K but also ppR_M. The spectrum was recorded at −100 °C (curve 1). The production of ppR_M at −80 °C is due to the thermal conversion of a part of ppR_K which is photochemically produced from the original ppR. Therefore, if the thermal conversion at pH 7.2 proceeds in the order of $ppR_K \rightarrow ppR_L \rightarrow ppR_M$, there must be some contribution of the absorption spectrum of ppR_L in curve 1. Next, this mixture was irradiated with >580-nm light at −100 °C, by which curve 1 was converted to curve 2. ppR_M was not changed, but ppR_K was converted back to ppR by this irradiation. Here, we have two possible interpretations if we assume the existence of ppR_L at pH 7.2: (1) ppR_L absorbs >580-nm light and is converted to the original ppR, and (2) ppR_L does not change under >580-nm light irradiation.

For the first possibility, if the putative ppR_L is present, curve 1 must be a composite of the difference absorption spectra of ppR_K, ppR_L, and ppR_M relative to that of the original ppR. Curve 2, however, is the difference spectrum of only ppR_M relative to ppR because ppR_K and ppR_L should be photoconverted to ppR. Therefore, the difference spectrum between curves 1 and 2 (curve 3 in Figure 6b) must be the difference spectrum of ppR_K and ppR_L relative to ppR. But curve 3 was almost identical with the difference spectrum between ppR_K and ppR at pH 7.2 (curve 4 in Figure 6b), which was obtained by irradiation of the photo-steady-state mixture composed of ppR and ppR_K with >580-nm light at −100 °C. Therefore, we can exclude the first possibility.

For the second possibility, curve 2 in Figure 6a must be the difference spectrum of a mixture of ppR_L and ppR_M relative to ppR. On the other hand, we obtained the difference spectrum between ppR_M and ppR at pH 9.0 (curve 8 in Figure 4 was replotted using the spectrum of unirradiated ppR sample as the baseline). This difference spectrum (curve 5 in Figure 6c) was almost identical with curve 2′ (Figure 6c: same as curve 2 in Figure 6a), indicating that the second possibility is also unlikely. Hence, neither possibility can explain the experimental results, which means that the assumption of the presence of ppR_L is not valid. We, therefore, concluded that ppR_L was absent at pH 7.2 as well as at pH 9.0. On the basis of these described results, we propose the photocycle of ppR as in Scheme I.

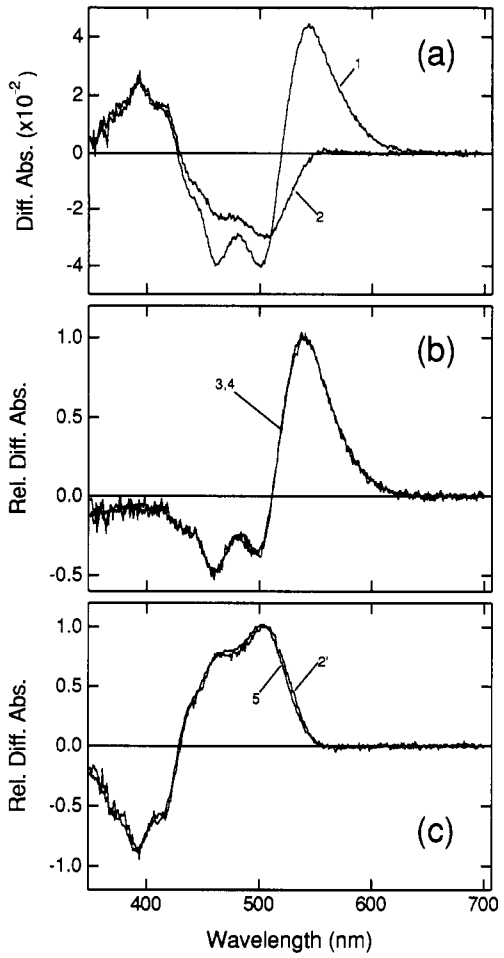


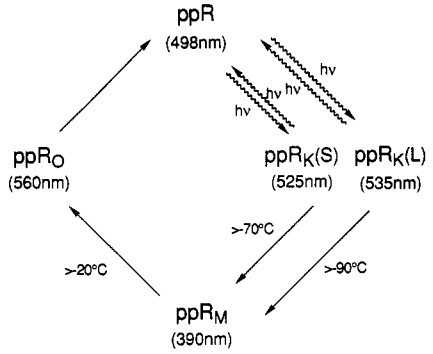
FIGURE 6: Absence of ppR_L at pH 7.2 as well as at pH 9.0. (a) The difference spectral changes of ppR at pH 7.2 by irradiation. The absorption spectrum of the ppR sample (pH 7.2) at −100 °C was used as the baseline. After warming to −80 °C, the sample was irradiated with 436-nm light for 320 s to form a mixture of ppR_K and its thermal product (ppR_m and putative ppR_L). After recording the spectrum at −100 °C (curve 1), it was irradiated with >580-nm light for 320 s for conversion of the ppR_K to the original ppR (curve 2). All spectra were recorded at −100 °C. (b) Examination of the formation of the putative ppR_L on the supposition that ppR_L absorbs >580-nm light. Since the putative ppR_L in curve 1 can be converted to the original ppR by >580-nm light, curve 2 should be the difference spectrum between the original ppR and ppR_M. Curve 3 is the difference spectrum between curve 1 [ppR_K + (ppR_L) + ppR_M − ppR] and curve 2 (ppR_M − ppR) in panel a. Curve 4 is a difference spectrum before and after irradiation of a mixture of ppR and ppR_K at pH 7.2 with >580-nm light at −100 °C. Curves 3 and 4 were normalized at 540 nm. Since they are in fairly good agreement with each other, we conclude that the putative ppR_L is absent. (c) Further examination of formation of the putative ppR_L on the supposition that the ppR_L does not absorb >580-nm light. Since the putative ppR_L in curve 1 cannot be converted by >580-nm light, curve 2′ (same as curve 2 in panel a) should be the difference spectrum of a mixture containing ppR_L and ppR_M from the original ppR. Curve 5 shows the difference spectrum between ppR_M and the original ppR at pH 9.0, which was calculated by subtracting the spectrum of the original ppR from curve 8 in Figure 4. These two curves were normalized at 505 nm. Their fairly good agreement with each other meant the absence of the putative ppR_L.

Discussion

We have shown that the reaction between the original ppR and ppR_K is photoreversible as true of other retinal proteins. Unlike pR, no hyposochromic photoproduct corresponding to P480 of pR (Imamoto et al., 1991) was detected by irradiation of the original ppR at liquid-nitrogen or liquid-helium temperature.

Although biphasic kinetics derived from the conversion process from ppR_K to ppR (Figure 3b) could be explained by the photoselection (Lewis et al., 1989), the difference in thermal stability between the two components of K intermediate is not explained by this mechanism. Thus it is clear that ppR_K is composed of two components [ppR_K(L) and ppR_K(S)] and that ppR_K(L) is thermally more unstable than ppR_K(S). From Figures 4 and 5b, the difference absorption maxima of ppR_K(L) and ppR_K(S) were estimated to be about 535 and 525 nm, respectively. Presence of two components of ppR_K strongly suggests that, after photoisomerization of the chromophore of ppR, the Schiff base region of the chromophore goes to its binding site having wider space to accommodate slightly variable chromophore conformations.

Scheme I: Photocycle of ppR[a]



[a] Wavy lines, photochemical reactions; smooth curves, thermal reactions. For details, see the text.

Several years ago it was reported that bathorhodopsin which corresponds to K intermediate of bR was also a mixture of two components, each of which was converted to its own lumi- and metarhodopsins (Sasaki et al., 1980). Therefore, whether or not the two kinds of ppR_K have their own ppR_M is interesting. In the conversion process from ppR_K to ppR_M (Figure 4), the positive peak indicating the formation of ppR_M also appears to shift to a shorter wavelength. This suggests that ppR_M may also be a mixture of two components. Further detailed experiments on this are necessary.

Like pR (Imamoto et al., 1991), ppR lacks the L-like intermediate in the photocycle under low temperature. This fact should be noted because all the photosensitive retinal proteins (rhodopsins, bR, hR, and sR) examined so far have their own L (lumi-) intermediate. In rhodopsin, changes in opsin conformation near the β-ionone ring region of the chromophore are essential for conversion of bathorhodopsin to lumirhodopsin (Shichida, 1986; Yoshizawa et al., 1987; Okada et al., 1991), while no experiment has focused on changes of opsin conformation near the β-ionone ring in the bacterial retinal proteins. Assuming that similar changes take place in the thermal decay of ppR_K as in that of rhodopsin, the lack of L-like intermediate of ppR suggests that the change in opsin conformation near the β-ionone ring is not a limiting factor in the conversion of ppR_K in ppR system. It is, however, noted that visual pigments contain a 6*S*-*cis*-chromophore whereas bacteriorhodopsin have a 6*S*-*trans*-chromophore (Harbison et al., 1985), although we have no information about C6–C7 bond of chromophore of pR and ppR.

The absolute absorption spectrum of pR has a peak at 487 nm and a shoulder at about 460 nm. Appearance of the shoulder is thought to originate from the fixation of the β-ionone ring and its side chain of the chromophore (Takahashi et al., 1990). ppR also has a shoulder at 470 nm. In addition, both pigments lack the L-like intermediates. The presence of a shoulder in the absolute absorption spectrum may, therefore, be related to the absence of L-like intermediates.

When ppR_M was cooled to −100 °C, the absorption spectrum of ppR_M displayed a prominent vibrational structure having a main peak at about 390 nm and shoulders at 370 and 416 nm (Figures 4, 5, and 6). Shoulders are also found in the S_{373} intermediate from sR (Bogomolni & Spudich, 1982).

Very recently, a difference absorption spectrum of a photoreceptor for phototaxis of *Chlamydomonas* was reported (Beckmann et al., 1991); its shape and absorption maximum were similar to those of ppR. The photoreceptor of *Chlamydomonas* was thought to be a progenitor of visual pigments in eukaryotes. Study of the molecular evolution of these photoreceptors may thus be of interest.

References

Bivin, D. B., & Stoeckenius, W. (1986) *J. Gen. Microbiol. 132*, 2167–2177.

Bogomolni, R. A., & Spudich, J. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6250.

Beckmann, M., & Hegemann, P. (1991) *Biochemistry 30*, 3692–3697.

Duschl, A., McCloskey, M. A., & Lanyi, J. K. (1988) *J. Biol. Chem. 263*, 17016–17022.

Duschl, A., Lanyi, J. K., & Zimanyi, L. (1990) *J. Biol. Chem. 265*, 1261–1267.

Harbison, G. S., Smith, S. O., Pardoen, J. A., Courtin, J. M., Lugtenburg, J., Herzfeld, J., Mathies, R. A., & Griffin, R. G. (1985) *Biochemistry 24*, 6955–6962.

Imamoto, Y., Shichida, Y., Yoshizawa, T., Tomioka, H., Takahashi, T., Fujikawa, K., Kamo, N., & Kobatake, Y. (1991) *Biochemistry 30*, 7416–7424.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lewis, J. W., Einterz, C. M., Hug, S. J., & Kliger, D. S. (1989) *Biophys. J. 56*, 1101–1111.

Ogurusu, T., Maeda, A., & Yoshizawa, T. (1984) *J. Biochem. (Tokyo) 95*, 1073–1082.

Okada, T., Kandori, H., Shichida, Y., Yoshizawa, T., Denny, M., Zhang, B.-W., Asato, A. E., & Liu, R. S. H. (1991) *Biochemistry 30*, 4796–4802.

Sasaki, N., Tokunaga, F., & Yoshizawa, T. (1980) *Photochem. Photobiol. 32*, 433–441.

Shichida, Y. (1986) *Photobiochem. Photobiophys. 13*, 287–307.

Shichida, Y., Imamoto, Y., Yoshizawa, T., Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1988) *FEBS Lett. 236*, 333–336.

Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1985) *FEMS Microbiol. Lett. 28*, 161–164.

Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K., & Spudich, J. L. (1990) *Biochemistry 29*, 8467–8474.

Tomioka, H., Takahashi, T., Kamo, N., & Kobatake, N. (1986a) *Biochem. Biophys. Res. Commun. 139*, 389–395.

Tomioka, H., Takahashi, T., Kamo, N., & Kobatake, N. (1986b) *Biochim. Biophys. Acta 884*, 578–584.

Tomioka, H., Otomo, J., Hirayama, J., Kamo, N., & Sasabe, H. (1990) *IVth International Conference on Retinal Proteins*, Santa Cruz, CA.

Wolff, E. K., Bogomolni, R. A., Scheerer, P., Hess, B., & Stoeckenius, W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7272–7276.

Yoshizawa, T., & Shichida, Y. (1982) *Methods Enzymol. 81*, 333–354.

Yoshizawa, T., Shinozawa, T., Shichida, Y., Matuoka, S., Ioshida, S., Kandori, H., & Sokabe, M. (1987) in *Retinal Proteins* (Hara, T., Ed.) pp 75–84, VNU Science Press, Zeist, The Netherlands.