Photoreaction Cycle of Phoborhodopsin Studied by Low-Temperature Spectrophotometry†

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ABSTRACT: The photochemical and subsequent thermal reactions of phoborhodopsin (pR_{490}), which mediates the negative phototaxis (phobic reaction) of *Halobacterium halobium*, were investigated by low-temperature spectrophotometry. At room temperature, the absorption spectrum of pR_{490} displayed vibrational structure with a maximum at 490 nm and a shoulder at 460 nm, which were remarkably sharpened by cooling, resulting in the appearance of two well-separated peaks. On irradiation of pR_{490} at −170 °C, a photo-steady-state mixture composed of pR_{490} and two photoproducts, P_{520} and P_{480}, was formed. P_{480} had an absorption maximum at 480 nm and thermally converted to pR_{490} above −160 °C, while P_{520} had an absorption maximum at 515 nm and thermally converted to P_{350}, the next intermediate, above −60 °C. Above −30 °C, P_{350} was converted to P_{530}, and then reverted to pR_{490}. P_{520}, P_{350}, and P_{530} may correspond to K, M, and O intermediates of bacteriorhodopsin, respectively, on the basis of their absorption spectra, but the intermediates corresponding to L and N intermediates were not observed. On the basis of these results, a new scheme of the photoreaction cycle of pR_{490} was presented.

Phoborhodopsin [(pR)[1] it is also called "sensory rhodopsin II (sRII)"] is one of four retinal proteins present in an archaebacterium, *Halobacterium halobium* (*H. halobium*). The other retinal proteins are bacteriorhodopsin (bR), halorhodopsin (hR), and sensory rhodopsin (sR). Among them, bR and hR are light-driven ion pumps to transport proton and chloride, respectively (Stoeckenius & Bogomolni, 1982; Lanyi, 1986), whereas pR and sR are photosensors for the phototaxis of *H. halobium* (Spudich & Bogomolni, 1988).

H. halobium swims with its flagella and occasionally changes the direction in the dark. The frequency of the directional change is decreased under yellow-red light and increased under UV-blue light (Spudich & Bogomolni, 1988). As a result, *H. halobium* accumulates in yellow-red light (positive phototaxis) and runs away from UV-blue light (negative phototaxis).

Since the sensitivity curve for the positive phototaxis has a maximum at about 590 nm, sensory rhodopsin (sR: λ_{max} = 587 nm) acts as a photoreceptive pigment. On the other hand, the negative phototaxis is driven by two distinct systems (Sundberg et al., 1986); one is mediated by a long-lived intermediate of sR, S_{373}, whose absorption spectrum (λ_{max} = 373 nm) is close to a sensitivity curve having a maximum at about 370 nm (Spudich & Bogomolni, 1984; Takahashi et al., 1985a,b), and the other has a maximal sensitivity at about 480 nm, which is derived from the absorption of photons by pR (Takahashi et al., 1985c; Tomioka et al., 1986).

The photoreactions of pR have been investigated by means of flash photolysis (Tomioka et al., 1986) and low-temperature spectrophotometry (Shichida et al., 1988). At least three intermediates were reported to be formed in its photoreaction cycle ($pR_{490} \xrightarrow{h\nu} P_{520} \rightarrow P_{350} \rightarrow P_{530} \rightarrow pR_{490}$, in which the original state of pR at room temperature is denoted as "pR_{490}" to distinguish it from its intermediates).

P_{520}, the earliest intermediate among them, was detected by irradiation of pR_{490} at −80 °C, whose difference absorption maximum before and after the irradiation was located at 520 nm (Shichida et al., 1988). The subsequent intermediates, P_{350} and P_{530}, were first discovered by flash photolysis at room temperature (Tomioka et al., 1986) and later confirmed by low-temperature spectrophotometry (Shichida et al., 1988). P_{530} thermally converted to pR_{490}. The lifetimes of P_{350} and P_{530} at room temperature were 100 and 300 ms, respectively. Low-temperature spectrophotometry confirmed the formation of both P_{350} and P_{530} from P_{520}, and the thermal conversion of P_{530} to pR_{490}. On the bases of their absorption characteristics, P_{520}, P_{350}, and P_{530} may correspond to K, M, and O intermediates of bR, respectively.

In the present paper, we have investigated photoreactions of pR_{490} at a lower temperature (−170 °C) than that used in the previous investigation (Shichida et al., 1988) in order to detect some of the intermediates. Afterward, the photoreaction of pR_{490} at low temperature was studied in detail to examine whether or not any other intermediates can be detected between the intermediates above. On the basis of the results

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[1] Abbreviations: pR, phoborhodopsin; bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; *H. halobium*, *Halobacterium halobium*; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; λ_{max}, absorption maximum in the visible region; UV, ultraviolet.

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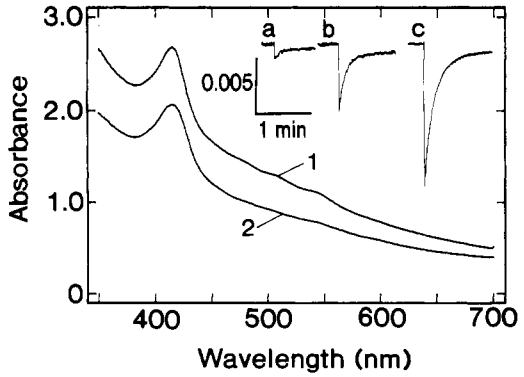

FIGURE 1: Absorption spectra of the samples used for the present experiments. Curve 1, a pR–membrane suspension of ON1-bW. Curve 2, the pR–66% glycerol sample used in the present low-temperature spectrophotometry. The amount of pR in the sample was estimated by recording the absorbance change at 480 nm after irradiation of the sample with yellow light (>480 nm) for 1 min at 0 °C (traces a–c). Trace, a, the pR–membrane suspension (curve 1). Trace b, a sample regenerated to pR by addition of *all-trans*-retinal to the pR–membrane suspension. Trace c, the pR–66% glycerol sample (curve 2).

obtained, a new photoreaction cycle of pR_{490} is presented. In addition, the absolute absorption spectra of pR_{490} and its primary intermediates are estimated. These results demonstrate that the photochemical properties of pR_{490} are strikingly different from those of other bacterial retinal proteins.

Materials and Methods

Preparation of the pR–Membrane Suspension. Mutant strains of *H. halobium*, Flx3-b1 (Takahashi et al., 1988) and ON1-bW (newly isolated from Flx3), which lack bR, hR, sR, and bacterioruberin were grown aerobically in a peptone medium (Oesterhelt & Stoeckenius, 1974) at 39 °C in the dark. pR–membranes isolated from the strain as described previously (Tomioka et al., 1986) were suspended in a buffer solution (20 mM HEPES–4.0 M NaCl, pH 7.0) and divided into two fractions for preparing samples.

Preparation of the pR–Opsin–50% Glycerol Sample. One of the fractions (membrane suspension) was pelleted by centrifugation (93000*g*, 20 min) and then washed with the buffer supplemented with 100 mM hydroxylamine in the light. Under these conditions, pR in the suspension was bleached to pR–opsin (apoprotein of pR) and retinal oxime. Then the membranes were washed 5 times with the buffer by centrifugation to remove free hydroxylamine. The pellet thus obtained was mixed with an equal volume of glycerol for lowering the turbidity of the suspension.

Preparation of the pR–66% Glycerol Sample. The other fraction was used for preparing a pR–66% glycerol sample. The strains used in the present experiments cannot produce enough *all-trans*-retinal; that is, only 20% of pR–apoprotein in the membrane was present as pR. In order to regenerate all the pR–opsin in the membrane to pR, *all-trans*-retinal dissolved in ethanol was added to the membrane suspension and then incubated for 24 h at room temperature (Figure 1). The regenerated pR in the suspension was pelleted by centrifugation and washed 8 times with the buffer by centrifugation. Then the pellet containing pR (1 volume) was suspended in glycerol (2 volumes) and homogenized by a Teflon homogenizer. To concentrate the pR in the mixture for optical measurements, the sample was extensively centrifuged (93000*g*, 14 h), and then the concentrated portion near the bottom of the centrifugation tube was collected. It was used as the sample for low-temperature spectrophotometry.

Low-Temperature Spectrophotometry. Absorption spectra were recorded with a Shimadzu Model MPS-2000 or a Hitachi Model 330 spectrophotometer, each of which was connected with a personal computer (PC9801F, NEC) through a GPIB or RS-232C circuit, respectively, for the purpose of storing and analyzing the absorption spectral data. The sample was put in the sample cell (2- or 5-mm light path) of a specially designed glass cryostat placed in the sample compartment of the spectrophotometer (Yoshizawa & Shichida, 1982). Temperatures of the sample were regulated by pouring liquid nitrogen into the coolant sink of the cryostat and monitored by a copper–constantan thermocouple attached to the sample cell holder of the cryostat. Opal glasses were set into both sample and reference beams of the spectrophotometer to compensate for light scattering by cracks of the sample formed by freezing. A 1-kW tungsten–halogen lamp (Sanko) was used as a light source for irradiating the sample, wavelengths of which were selected by using optical filters [blue light at 436 nm and a green light at 501 nm; interference filters (Nihonshinku, half-bandwidth = 2 nm), a blue-green light at 469 nm; a combination of an interference filter (KL47, Toshiba, half-bandwidth = 19 nm) and a glass cutoff filter (Y46, Toshiba), red (>580 nm), orange (>540 nm), and yellow (>480 nm) lights; glass cutoff filters (R60, O56, and Y50, respectively, Toshiba)].

Results and Discussion

Absorption Spectra of the Samples. Since the content of pR in the *H. halobium* cell was small, it was difficult to isolate pR from other proteins or carotenoids present in the cell membrane by such physical manipulations as sucrose flotation. The chemical purification methods such as column chromatography cannot be used because a suitable detergent which solubilizes pR molecules without denaturation has not yet been discovered. A typical example of the absorption spectra of the sample prepared for the present experiments is shown in Figure 1. The amount of pR in the sample was estimated by measurement of the absorbance change at 480 nm after irradiating the sample with yellow light (>480 nm) at 0 °C (traces a–c in Figure 1). The pR–membrane suspension of ON1-bW (curve 1 and trace a) contained pR and pR–opsin with a large amount of heme protein (λ_{max} = 415 and 560 nm). The pR–opsin in the pR–membrane suspension was regenerated to pR by adding *all-trans*-retinal (trace b). After being pelleted, it was mixed with 66% glycerol in the final concentration to lower the turbidity, followed by concentration by centrifugation (pR–66% glycerol sample; curve 2 and trace c). In the following experiments, the difference spectrum of the sample in the near-UV region could not be recorded because of the large absorbance by the impurities.

Absorption Spectrum of pR_{490} at Room Temperature. Owing to a large amount of colored impurities present in the pR–66% glycerol sample, we could not detect the absorption maximum of pR_{490}. Therefore, we estimated it by measuring the difference spectrum before and after regeneration of pR–opsin to pR_{490}. Namely, two optical cells filled with the pR–opsin–50% glycerol sample were set in the sample and reference sides of the sample compartment of the spectrophotometer for recording the base line. Then *all-trans*-retinal dissolved in ethanol was added to the pR–opsin in the cell of the sample side. In order to exclude the effect of the ethanol added to the pR–opsin, an equivalent volume of ethanol was added to the pR–opsin in the cell of the reference side. After incubation for 30 min at 20 °C in the dark, the spectrum was recorded (Figure 2a). The spectrum had an absorption maximum at 491 nm and a shoulder at about 460 nm, which were close to

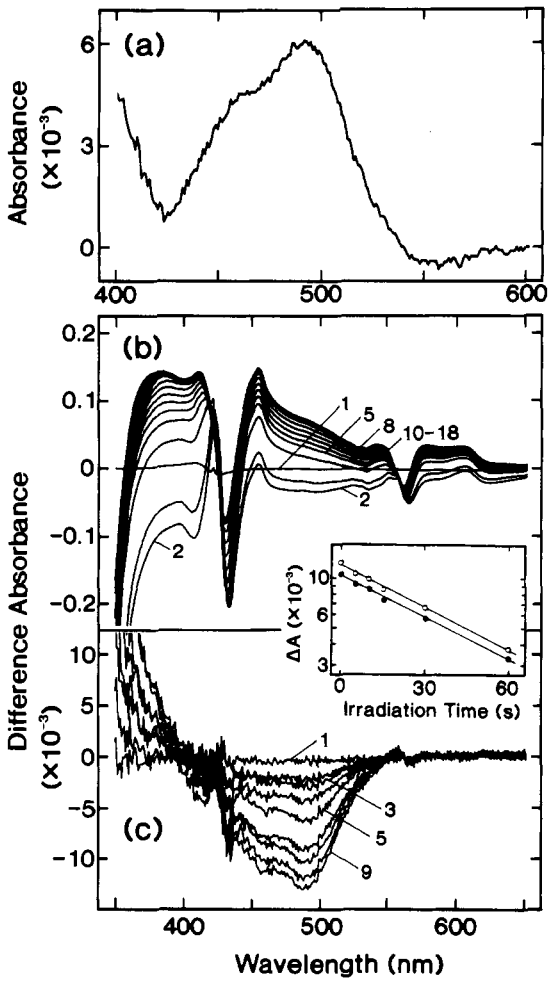

FIGURE 2: Estimation of the absorption spectrum of pR_{490}. (a) The pR–opsin–50% glycerol sample was mixed with *all-trans*-retinal in ethanol and then incubated for 30 min at 20 °C for regeneration of the pR–opsin to pR. The difference spectrum before and after the regeneration is shown (λ_{max} = 491 nm). (b) Spectral changes induced by reduction of the heme protein(s) in the pR–50% glycerol sample by sodium hydrosulfite. The pR–50% glycerol mixture (curve 1) was mixed with 2 mM sodium hydrosulfite and 10 mM hydroxylamine at 0 °C (curve 2). The sample was kept at 0 °C in the dark for 8 h until the reduction of heme protein(s) was completed. The spectra were recorded at intervals of 30 min (curves 3–18, respectively). (c) The photobleaching process of pR_{490} in the presence of 2 mM sodium hydrosulfite and 10 mM hydroxylamine. After the complete reduction, the final spectrum (curve 18 in panel b) was recorded as the base line (curve 1). Then it was irradiated with green light (501 nm) at 0 °C for a total of 5, 10, 15, 30, 60, 120, 240, and 480 s (curves 2–9, respectively) (λ_{max} = 489 nm). Inset: Decreases of the difference absorbances at 490 nm (open circles) and 460 nm (closed circles) were plotted against irradiation time.

the values previously reported (Takahashi et al., 1988, 1990). The difference spectrum of pR_{490} was also recorded by photobleaching of pR_{490} in the pR–50% glycerol sample as follows (Figure 2b,c).

Our preliminary experiments showed that pR_{490} itself was stable in the presence of 10 mM hydroxylamine at 0 °C. Then 10 mM hydroxylamine was added to the sample, inducing a large spectral shift owing to reduction of heme protein(s) present in the sample. Furthermore, the spectral shift due to the reduction was accelerated by irradiation of the sample. In order to record only the light-induced spectral shift of pR_{490}, the heme protein(s) in the sample was (were) completely reduced with sodium hydrosulfite ($Na_2S_2O_4$) prior to the photobleaching experiments. Namely, sodium hydrosulfite and hydroxylamine were added to the sample to give final concentrations of 2 and 10 mM, respectively, followed by incubation in the dark at 0 °C for 8 h until the spectrum showed no further shift (Figure 2b). After complete reduction, the sample was irradiated with a green light at 501 nm (Figure 2c). The difference spectrum before and after the complete bleaching of pR_{490} (curve 9 in Figure 2c; λ_{max} = 489 nm) was almost identical with that obtained by regeneration in the visible region (Figure 2a). The absorbance changes at the peak (490 nm, open circles) and the shoulder (460 nm, closed circles) in the photobleaching process were plotted against the irradiation time (Figure 2, inset). They displayed first-order kinetics with the same time constant. This fact indicates that not only the sample is composed of a single component but also the spectrum of pR_{490} displays vibrational structure. The spectrum obtained was in good agreement with the action spectrum of the phototactic behavior of the bacterium (Takahashi et al., 1988).

Photochemical Reactions of pR_{490} at −170 °C. Our previous paper on photochemical reactions of pR_{490} at −80 °C reported the formation of a bathochromic product called P_{520} whose difference absorption maximum relative to pR_{490} was located at 520 nm (Shichida et al., 1988). P_{520} may correspond to the K intermediate of bR according to the criterion of its absorption spectrum. On the basis of its thermal stability, however, it may correspond to the L intermediate. Thus, we examined whether or not a precursor of P_{520} is formed at −170 °C, a much lower temperature than that used in the previous investigation.

The experiments were started by recording a spectrum of the pR–66% glycerol sample as the base line (curve 1 in the inset of Figure 3a). Hence, all the spectra recorded were difference spectra between the photoproducts and pR_{490} (Figures 3 and 4). The main panels of Figures 3 and 4 show the absolute absorption spectra of the mixture of pR_{490} and its intermediates calculated by adding the difference spectra in the insets to the spectrum of pR_{490} at −170 °C (Figure 9a).

On irradiation of pR_{490} at −170 °C with blue light (436 nm), a broad positive band having a difference maximum at 520 nm and two sharp negative peaks at 480 and 460 nm were observed (Figure 3a, inset). Appearances of negative and positive bands are due to the conversion of pR_{490} and the formation of a bathochromic photoproduct, P_{520}, respectively. The main panel clearly demonstrates that the irradiation caused a red-shift of the absorption spectrum of pR_{490}. Prolonged irradiation formed a photo-steady-state mixture whose spectrum was not changed by further irradiation with blue light. It should be noted that an intersection point was located at 500 nm in the early stages of the irradiation and then shifted to 505 nm in the later stages (Figure 3a). These facts indicate that the photo-steady-state mixture produced by prolonged irradiation was composed of at least two photoproducts and pR_{490}. In fact, reirradiation of the photo-steady-state mixture with the red light (>580 nm) or the orange light (>540 nm) shifted the spectrum toward the base line (pR_{490}), but not completely (curve 15 in Figure 3b, inset); these spectral changes were mainly due to reversion of P_{520} to pR_{490}. Therefore, a photoproduct different from P_{520} was formed by prolonged irradiation with the blue light at −170 °C.

In order to establish the formation of the new photoproduct, pR_{490} was irradiated with yellow light (>480 nm) at −170 °C until no further spectral changes were observed (Figure 3c). The difference spectrum obtained (curve 6 in Figure 3c, inset) showed a small positive peak at about 470 nm and two negative peaks at 490 and 460 nm. The addition of the absorption spectrum of pR_{490} to the difference spectrum clearly indicated

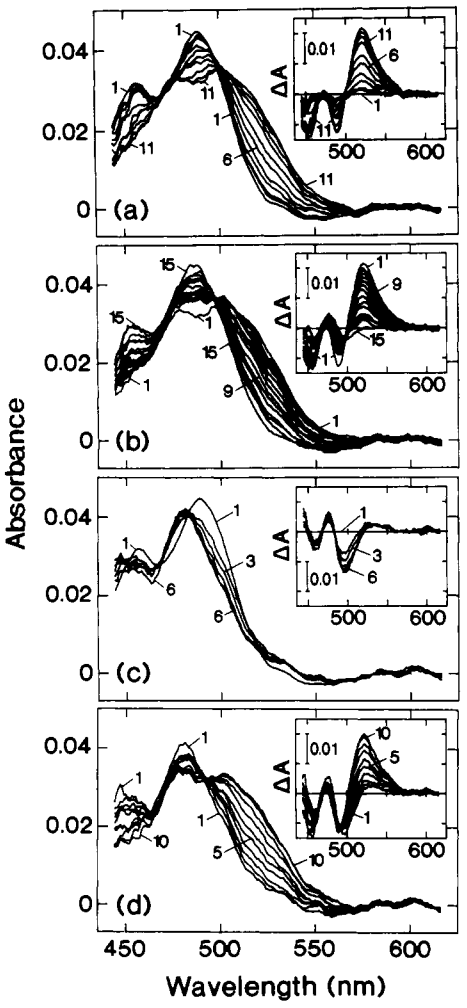

FIGURE 3: Photoreactions of pR_{490} at −170 °C. The difference spectra between pR_{490} and its photoproducts formed by irradiation at −170 °C with color light(s) are shown in insets. In the main panels, the absorption spectra were composed by adding the difference spectra to the spectrum of pR_{490} (Figure 9a). They demonstrate the spectral changes upon irradiation. The curve numbers in the main panels correspond to those of the difference spectra presented in the insets. (a) Inset: The spectrum of the pR–66% glycerol sample was recorded as the base line (curve 1). Then the sample was irradiated with blue light (436 nm) at −170 °C for a total of 5, 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 s (curves 2–11, respectively) until a photo-steady-state mixture was formed. Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. It is clearly shown that pR_{490} was converted to a bathochromic photoproduct, P_{520}, by light. (b) Inset: After irradiation of the pR–66% glycerol sample with blue light (curve 1, same as curve 11 in panel a), it was irradiated with red light (>580 nm) for a total of 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120, 10240, and 20480 s (curves 2–14, respectively) and then irradiated with orange light (>560 nm) for 160 s (curve 15). Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. P_{520} was converted not only to pR_{490} but also to another photoproduct, P_{480}. (c) Inset: After the spectrum of the pR–66% glycerol sample was recorded as the base line (curve 1), it was irradiated with yellow light (>480 nm) for a total of 5, 10, 20, 40, and 80 s (curves 2–6, respectively). Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. pR_{490} was converted to a hypsochromic photoproduct whose absorption maximum was located at 480 nm (P_{480}). (d) Inset: The sample mainly containing P_{480} (curve 1, same as curve 6 in panel c) was irradiated with blue light at −170 °C for a total of 5, 10, 20, 40, 80, 160, 320, 640, and 1280 s (curves 2–10, respectively) until a photo-steady-state mixture (curve 10) was formed. Curve 10 was almost identical with curve 11 in panel a, indicating that pR_{490}, P_{480}, and P_{520} were perfectly interconvertible by light at −170 °C. Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. P_{480} was converted to P_{520} with light.

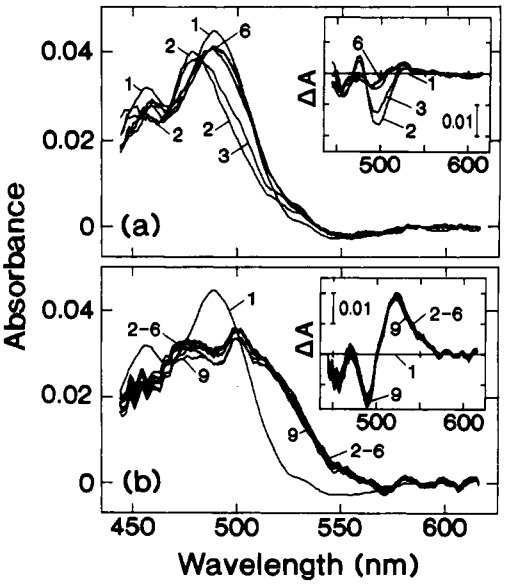

FIGURE 4: Thermal reactions of P_{520} and P_{480}. (a) Inset: After the spectrum of the pR–66% glycerol sample was recorded as the base line (curve 1), the photo-steady-state mixture mainly containing P_{480} was produced by yellow light irradiation (>480 nm) at −170 °C (curve 2). It was successively warmed to −160, −150, −140, and −130 °C, and after each warming, it was recooled to −170 °C for spectral measurement (curves 3–6, respectively). Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. (b) Inset: After the spectrum of the pR–66% glycerol mixture was recorded as the base line (curve 1), the sample mainly containing P_{520} formed by blue light irradiation (436 nm) at −170 °C (curve 2) was successively warmed to −160, −150, −140, −130, −120, −110, and −90 °C, and after each warming, it was recooled to −170 °C for spectral measurement (curves 3–9, respectively). Main panel: Spectra composed by adding the difference spectra in the inset to the spectrum of pR_{490}. Only slight spectral change took place.

that the absorption maximum of this photoproduct was located at 480 nm (curve 6 in Figure 3c). We will call this photoproduct P_{480}. P_{480} showed a red-shift upon irradiation with the blue light (Figure 3d). The final curve (curve 10) was almost identical with that of the photo-steady-state mixture formed by irradiation of pR_{490} with the same light (curve 11 in Figure 3a), indicating that these three components, pR_{490}, P_{520}, and P_{480}, were perfectly photoreversible at −170 °C.

Thermal Stabilities of P_{480} and P_{520}. The thermal reactions of P_{480} or P_{520} were investigated by stepwise warming of the photo-steady-state mixtures mainly containing P_{480} or P_{520} from −170 to −130 or −90 °C, respectively (Figure 4). The mixture mainly containing P_{480} (curve 2 in Figure 4a, inset; same as curve 6 in Figure 3c) was warmed to a desired temperature and then recooled to −170 °C for recording the spectra. Above −160 °C, the spectrum of P_{480} was successively shifted toward the base line (pR_{490}) (curves 3–6 in Figure 4a, inset). The final curve (curve 6) was not in agreement with the base line. This would be due to the presence of a small amount of P_{520} in the mixture because curve 2 had a positive band at about 520 nm which still remained on warming of the sample to −130 °C. The thermal reaction formed a clear isosbestic point at 481 nm, indicating that P_{480} was directly converted to pR_{490} above −160 °C.

The thermal reactions of P_{520} were studied in a similar manner (Figure 4b). The sample containing mainly P_{520} (curve 2; same as curve 11 in Figure 3a) was warmed in a stepwise manner from −170 to −90 °C, but no significant change of the spectra was observed (curves 3–9). This fact indicates that P_{520} was thermally stable at least below −90 °C and it would be the same species as P_{520} formed by irradiation at −80 °C

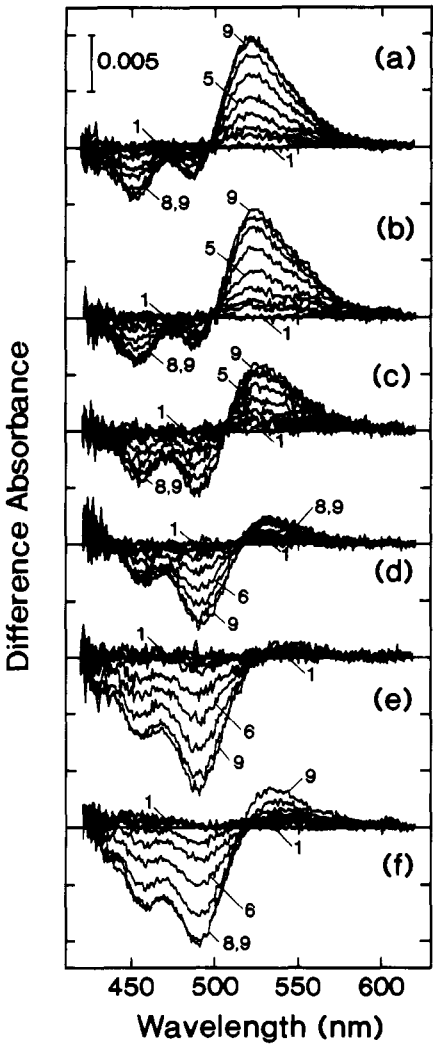

FIGURE 5: Photochemical reactions of pR_{490} at various temperatures. In every panel, the spectrum of the pR-66% glycerol sample was recorded as the base line (curve 1) followed by irradiations with blue light (436 nm) for a total of 5, 10, 20, 40, 80, 160, 320, and 640 s (curves 2–9, respectively). Irradiations were performed at −80 (panel a), −70 (b), −60 (c), −50 (d), −40 (e), and −30 °C (f).

as reported previously (Shichida et al., 1988).

The thermal conversion process of P_{520} was further examined above −90 °C by stepwise warming of the mixture mainly containing P_{520}. However, we failed to record precise absorption spectral changes because of a large deviation of the base line of the spectra by the warming, probably owing to a large amount of impurities in the pR sample. Thus, we tried to irradiate the pR–66% glycerol sample with blue light (436 nm) at various temperatures ranging from −80 to −30 °C (Figure 5).

As reported previously (Shichida et al., 1988), the spectrum of pR_{490} was red-shifted by irradiation with the blue light (436 nm) at −80 °C, owing to the formation of P_{520} (Figure 5a). In this spectral change, a clear isosbestic point was present at 495 nm, indicating that only P_{520} was formed at −80 °C. Similar spectral changes were observed at −70 °C (Figure 5b). At −60 °C (Figure 5c) or −50 °C (Figure 5d), however, the positive peak at 520 nm decreased in comparison with that at −80 or −70 °C, while the negative peaks increased, indicating further conversion of pR_{490}. At −40 °C, almost no positive peak was observed in the measured wavelength region, but only the negative peaks at 490 and 460 nm were observed (Figure 5e). These results suggest that P_{520} is thermally unstable above −60 °C and is completely converted to the next

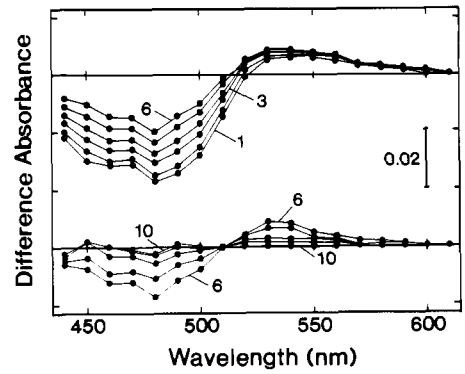

FIGURE 6: Thermal conversions of P_{350} to pR_{490} through P_{530}. Time courses of absorbance changes of pR_{490} were recorded after irradiation of the pR–66% glycerol sample with yellow light (>480 nm) for 1 min at −10 °C at an interval of 10 nm in the range from 610 to 440 nm, and the absorbance changes at times described below were plotted. (a) The early recovery process to pR_{490}. The absorbance changes at 10, 20, 40, 60, 90, and 120 s after irradiation (curves 1–6, respectively) were plotted. (b) The late recovery process to pR_{490}. The absorbance changes at 120, 240, 360, 480, and 600 s after irradiation (curves 6–10, respectively) were plotted.

intermediate during irradiation at −40 °C. Since curve 9 in Figure 5e seems almost identical with the absorption spectrum of pR_{490} (Figure 2a) except for its sharpening by cooling, the next intermediate has little absorbance in the wavelength region from 440 to 650 nm. This observation suggests that the next intermediate is P_{350} which had been discovered by flash photolysis at room temperature (Tomioka et al., 1986). At −30 °C, another bathochromic photoproduct (P_{530}) appeared (Figure 5f), which continued to accumulate even after the photoconversion of pR_{490} in the sample was saturated (curves 8 and 9 in Figure 5f). P_{530} would be formed by thermal conversion of P_{520} through P_{350} which was somewhat unstable at −30 °C.

The recovery of pR_{490} from P_{350} through P_{530} was confirmed by measurements of the time courses of the absorbance changes in detail after irradiating the sample with yellow light (>480 nm) for 1 min at −10 °C (Figure 6). The absorbance changes were obtained by measuring the difference absorbances before and at a proper time (10–600 s) after the irradiation. The difference absorbances which were measured at an interval of 10 nm ranging from 610 to 440 nm were plotted against wavelengths for forming difference spectra (Figure 6). Immediately after the irradiation, the absorbances at longer wavelengths than 520 nm increased, but those at shorter wavelengths decreased (curve 1). This indicates that not only P_{350} but also P_{530} was formed during the irradiation. Then the absorbances at all the wavelengths were increased in the first 120 s (curves 1–6), indicating that P_{350} was thermally converted to P_{530}, some of which was further converted to pR_{490}. At 120 s after the irradiation, the thermal conversion proceeded with a clear isosbestic point at 510 nm (curves 6–10), indicating that P_{350} decayed almost completely to P_{530}, which was further converted to pR_{490}. The time course at 510 nm was due to the decay of P_{350} to P_{530}, and the time courses at 480 and 530 nm were explained by the reaction composed of two sequential processes ($P_{350} \rightarrow P_{530} \rightarrow pR_{490}$). The lifetimes of P_{350} and P_{530} at −10 °C were estimated to be 50 and 160 s, respectively, by the nonlinear least-squares method.

Possibility of the Existence of L- and N-Like Intermediates. The experimental results described above showed the formation of at least three intermediates in the photoreaction cycle of pR_{490}, in which P_{480} was neglected because it was regarded as a side product formed from pR_{490} by two successive photochemical reactions (see Characteristics of pR). On the basis

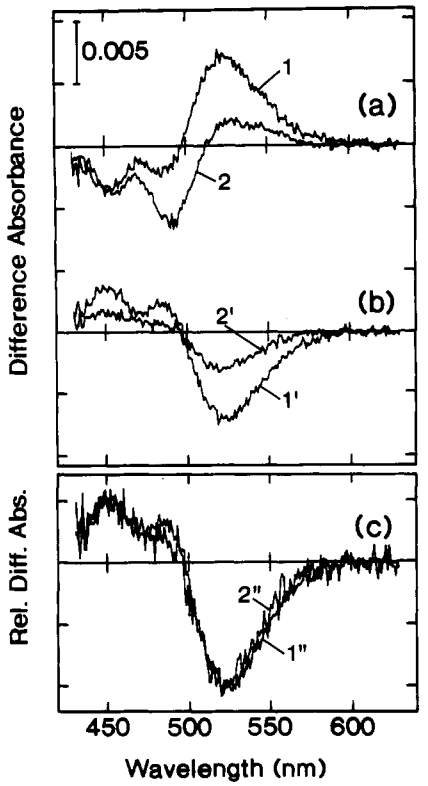

FIGURE 7: Spectral examination of a putative bathochromic intermediate corresponding to L. After the spectrum of the pR–66% glycerol sample was recorded as the base line at −80 °C, the sample was irradiated with blue light (436 nm) for 640 s at −80 °C, followed by recording the difference spectrum before and after irradiation (curve 1 in panel a). Then the sample was irradiated with orange light (>540 nm) for 80 s, followed by recording the difference spectrum before and after irradiation (curve 1′ in panel b). Next, another aliquot similarly irradiated at −80 °C was incubated at −60 °C for 30 min and recooled to −80 °C, followed by recording the difference spectrum before and after these manipulations (curve 2 in panel a). Then this sample was irradiated with orange light (>540 nm) for 80 s, and the difference spectrum before and after irradiation was recorded (curve 2′ in panel b). When curves 1′ and 2′ were normalized at their peaks (curves 1″ and 2″ in panel c), they were almost identical with each other, indicating the photoconversion due to the same species, P_{520}.

of the absorption spectra, P_{520}, P_{350}, and P_{530} may correspond to K, M, and O intermediates of bR, respectively, as follows:

$$pR_{490} \xrightarrow{h\nu} P_{520} \rightarrow ? \rightarrow P_{350} \rightarrow ? \rightarrow P_{530} \rightarrow pR_{490}$$

$$bR_{583} \xrightarrow{h\nu} K_{626} \rightarrow L_{543} \rightarrow M_{418} \rightarrow N_{560} \rightarrow O_{640} \rightarrow bR_{583}$$

The subscripts of bR, K, L, and M indicate the λ_{max} values observed by low-temperature spectrophotometry (Iwasa et al., 1980), and those of N and O at room temperature photolysis (Drachev et al., 1987; Lozier et al., 1975). The comparison of the photoreaction cycle of pR_{490} with that of bR_{583} further suggests the possible existence of intermediates of pR_{490} corresponding to L_{543} and N_{560}. The fact that we could not detect any intermediate on the conversion process from P_{350} to P_{530} would be reasonable because the thermal stabilities of P_{350} and P_{530} were very close to each other. On the other hand, L-like intermediate (pR_L) should be detected under our experimental conditions if it is present. So we have tried to look for pR_L in the temperature region from −70 to −40 °C because P_{520} (K) was stable below −60 °C and completely converted to P_{350} at −40 °C (see Figure 5). Experimentally, photoreactions of the intermediate present in the mixture produced by incubation at −60 °C after irradiation of pR_{490} at −80 °C were compared with that of P_{520} (Figure 7) as follows: An

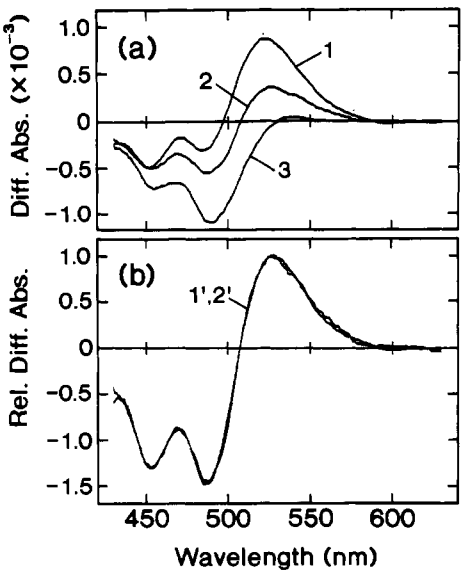

FIGURE 8: Simulation analysis for the demonstration of the lack of pR_L. We tried to clarify whether the difference spectrum obtained by irradiation with 436-nm light at −60 °C (curve 9 in Figure 5c) contained components other than pR_{490}, P_{520}, and P_{350}. (a) The difference spectra obtained by irradiation with blue light (436 nm) at −80, −60, and −40 °C are shown (curves 1, 2, and 3, respectively). They are the same as curves 9 in Figure 5a,c,e, respectively. (b) Curve 1′ is identical with curve 2. Curve 2′ was reconstituted by the sum of 44% of curve 1 and 40% of curve 3. Curve 2′ was in good agreement with curve 1′. Since curve 1 was the difference between pR_{490} and P_{520} and curve 3 was the difference between pR_{490} and P_{350}, curve 2 should not contain the contribution of the other component than pR_{490}, P_{520}, and P_{350}.

aliquot of the pR–66% glycerol sample was irradiated with 436-nm light at −80 °C until the photo-steady-state mixture composed of pR_{490} and P_{520} was formed, and the difference spectrum before and after the irradiation was recorded (curve 1 in Figure 7a). Then the sample was irradiated with orange light (>540 nm) to convert P_{520} to pR_{490}, and the difference spectrum before and after irradiation was recorded (curve 1′ in Figure 7b). Another aliquot of the same sample was also irradiated with 436-nm light at −80 °C to produce the photo-steady-state mixture (pR_{490} and P_{520}), followed by incubation at −60 °C for 30 min for causing the thermal reaction of P_{520}. After the sample was recooled to −80 °C, the difference spectrum between this sample and the original sample was recorded (curve 2 in Figure 7a). Then it was irradiated with orange light (>540 nm), followed by recording the difference spectrum before and after irradiation (curve 2′ in Figure 7b). In panel c, curve 1′ was compared with curve 2′ after normalizing at their peaks at 520 nm (curves 1″ and 2″, respectively). These two spectra showed almost identical shapes, indicating that the bathochromic photoproduct present in the sample incubated at −60 °C (curve 2 in panel a) was P_{520}. From this result, it became clear at least that the putative pR_L was not an intermediate which is photoconvertible and has a red-shifted spectrum. There are still two possibilities: the first is that the pR_L did not absorb the orange light (>540 nm), and the second is that pR_L did not convert on photon absorption. To explore these possibilities, a simulation analysis was carried out (Figure 8).

The difference spectrum between pR_{490} and P_{520} (curve 9 in Figure 5a) and that between pR_{490} and P_{350} (curve 9 in Figure 5e) obtained by irradiation of pR_{490} at −80 and −40 °C, respectively, were redrawn as curves 1 and 3 in Figure 8a. If the difference spectrum obtained by irradiation at −60 °C (curve 2: same as curve 9 in Figure 5c) can be recon-

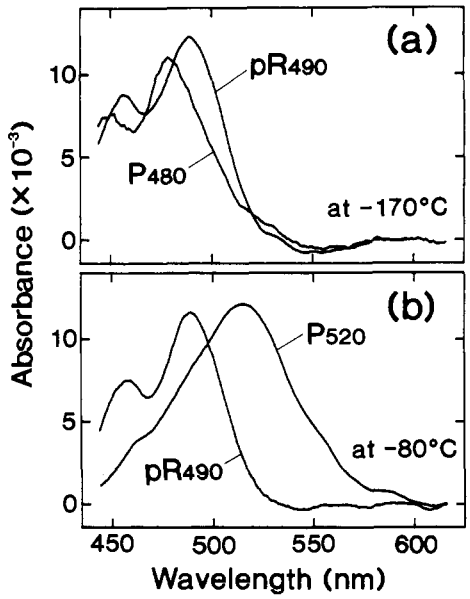

FIGURE 9: Absolute absorption spectra of pR_{490}, P_{480}, and P_{520} at low temperatures. (a) The absorption spectra of pR_{490} (λ_{max} = 489 nm) and P_{480} (λ_{max} = 479 nm) at −170 °C. (b) The absorption spectra of pR_{490} (λ_{max} = 489 nm) and P_{520} (λ_{max} = 515 nm) at −80 °C. For estimation criteria, see the text.

stituted by a combination of a certain amount of curve 1 and curve 3, it may be concluded that the mixture obtained at −60 °C (curve 2) was composed of only pR_{490}, P_{520}, and P_{350}, and does not contain pR_L. Indeed, the difference spectrum reconstituted from 44% of curve 1 and 40% of curve 3 (curve 2′ in Figure 8b) was superimposable on curve 2 in Figure 8a (curve 1′ in Figure 8b). Therefore, pR has no L-like intermediate in its photoreaction cycle.

Absorption Spectra of pR_{490} at Low Temperatures. Since the absorption spectra of retinal proteins generally change on cooling from room temperature to low temperatures, the spectrum of pR_{490} at low tempertures was estimated, which is essential for calculation of the spectra of its intermediates. As described, we have measured the spectrum of pR_{490} at 20 °C using the regeneration or photobleaching technique in the presence of hydroxylamine. However, applications of these techniques to estimate a low-temperature spectrum were impossible because pR neither regenerates nor photobleaches in the frozen medium at low temperatures, where its intermediates were stable. Therefore, the absorption spectrum of pR_{490} at low temperatures was estimated from the difference spectra between pR_{490} and its intermediates. Since the absorption maximum of P_{350} is located at about 350 nm, it is reasonable to assume that P_{350} has absorbance only in the range below 450 nm. Therefore, the difference spectrum above 450 nm between pR_{490} and P_{350} obtained by irradiating the pR–66% glycerol sample at −40 °C (Figure 5e) would be identical with the absolute spectrum of pR_{490} at −40 °C.

The absorption spectrum of pR_{490} at −80 °C was estimated by measuring the difference spectrum between pR_{490} and P_{350} at −80 °C (Figure 9b) as follows: The pR–66% glycerol sample was subjected to spectral measurement at −80 °C (the first spectrum), followed by warming to −40 °C, irradiating with blue light (436 nm) for formation of P_{350}, and cooling to −80 °C for measuring the spectrum. The last spectrum was subtracted from the first spectrum. This difference spectrum corresponding to the absorption spectrum of pR_{490} at −80 °C is shown in Figure 9b.

This procedure could not be applied to the estimation of the spectrum of pR_{490} at −170 °C because of a spectral deviation due to formation of various amounts of cracks in the sample on cooling to −170 °C. Thus, the spectrum was estimated as follows: The change of the spectrum of P_{520} on cooling from −80 to −170 °C is much smaller than that of pR_{490} as evidenced by close similarity in the difference spectrum at wavelengths longer than 540 nm between curve 9 in Figure 5a and curve 11 in the inset of Figure 3a. The amount of P_{480} in the photo-steady-state mixture formed at −170 °C (curve 11 in Figure 3a, inset) was negligible because little spectral change was observed on warming from −170 to −130 °C, by which P_{480} should convert to pR_{490} (curves 2–6 in Figure 4b, inset). Then we calculated the spectral difference between curve 9 in Figure 5a and curve 11 in the inset of Figure 3a after normalizing the amount of P_{520} contributed in the spectra. The difference spectrum thus obtained was regarded as the cooling effect on the spectrum of pR_{490} from −80 to −170 °C. Thus, the spectrum of pR_{490} at −170 °C was calculated by adding it to the spectrum of pR_{490} at −80 °C (Figure 9a).

Absorption Spectrum of P_{480}. Since the irradiation of pR_{490} with yellow light caused an almost complete conversion of pR_{490} to P_{480} (Figure 3c), the spectrum of P_{480} (curve 6 in Figure 3c) was calculated by adding the difference spectrum between pR_{490} and P_{480} obtained by yellow light irradiation at −170 °C (curve 6 in Figure 3c, inset) to the spectrum of pR_{490} (Figure 9a).

Absorption Spectrum of P_{520}. To calculate the absolute absorption spectrum of P_{520}, the ratio of the amount of pR_{490} to that of P_{520} in the photo-steady-state mixture composed of them must be estimated. Since the shape of the absorption spectrum of pR_{490} was almost identical with the action spectrum of the phototaxis of bacterium (Takahashi et al., 1988), it is reasonable to assume that the quantum yield of pR_{490} is independent of wavelength. If the quantum yield of P_{520} is also independent of wavelength, the ratio can be estimated as follows:

pR_{490} in the pR–66% glycerol sample was irradiated at −80 °C with blue-green light at 469 nm or green light at 501 nm until each photo-steady-state mixture was formed. In the photo-steady-state mixture produced by the blue-green light irradiation, the equation can be derived:

$$(1 - x)A(pR_{490}, 469\ nm) = \phi x A(P_{520}, 469\ nm)$$

where x is the relative amount of P_{520} in the photo-steady-state mixture, ϕ is the relative quantum yield of P_{520} to pR_{490}, and $A(pR_{490}, 469\ nm)$ and $A(P_{520}, 469\ nm)$ are the absorbance of pR_{490} and P_{520} at 469 nm, respectively. A similar equation can be derived on the photo-steady-state mixture produced by green light irradiation:

$$(1 - x')A(pR_{490}, 501\ nm) = \phi x' A(P_{520}, 501\ nm)$$

where x' is the relative amount of P_{520} in the photo-steady-state mixture by green light irradiation.

On the other hand, the following equations are derived from the difference spectra before and after blue-green light (469 nm) irradiation:

$$\Delta A(469\ nm) = xA(P_{520}, 469\ nm) - xA(pR_{490}, 469\ nm)$$

$$\Delta A(501\ nm) = xA(P_{520}, 501\ nm) - xA(pR_{490}, 501\ nm)$$

where $\Delta A(469\ nm)$ and $\Delta A(501\ nm)$ are the difference absorbances at 469 and 501 nm of the difference spectrum, respectively. Among the values presented in the equations described above, the following values were experimentally determined: x'/x = 0.79, $A(pR_{490}, 469\ nm)$ = 0.0065, $A(pR_{490}, 501\ nm)$ = 0.0086, $\Delta A(469\ nm)$ = −0.0017, and

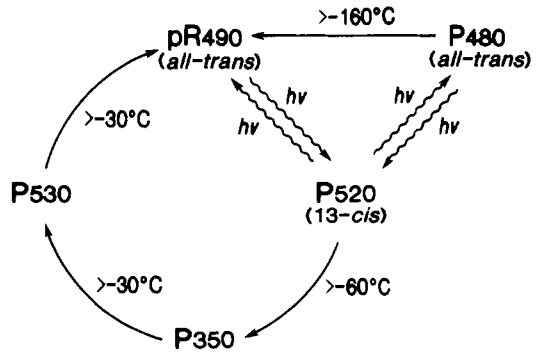

FIGURE 10: Photoreaction cycle of pR_{490} at low temperatures. Wavy lines, photoreactions; solid lines, thermal reactions. P_{520}, P_{350}, and P_{530} correspond to K, M, and O intermediates of bR, respectively, while this scheme lacks the intermediates corresponding to L or N. The absence of the L intermediate was definitely confirmed by the experiments present in this work, whereas our experiments did not support the presence of the N intermediate of pR.

$\Delta A(501\text{ nm}) = 0.0011$. Then the values for x and ϕ were calculated to be 0.70 and 0.68, respectively.

The absorbance of P_{520} at N nm can be written as

$$A(P_{520}, N\text{ nm}) = A(pR_{490}, N\text{ nm}) + \Delta A(N\text{ nm})/0.70$$

Thus, the absorption spectrum of P_{520} was calculated and shown in Figure 9b. The absorption maximum was located at 515 nm, and the fine structure observed in the spectrum of pR_{490} or P_{480} disappeared.

Characteristics of pR. The scheme of the photoreaction cycle of pR_{490} derived from low-temperature spectrophotometry is shown in Figure 10. The absorption maximum of pR_{490} lies at a much shorter wavelength than those of the other bacterial retinal proteins (bR, 568 nm; hR, 578 nm; sR, 587 nm). Nevertheless, pR_{490} has an *all-trans*-retinal as its chromophore like the other retinal proteins. Since the wavelength of absorption maximum of a retinal protein is regulated by steric and electrostatic interactions between the retinylidene chromophore and the amino acid residues present in the retinal binding site of the protein moiety, the environment around the retinylidene chromophore of pR_{490} must be different from the others. Additionally, pR_{490} has several peculiarities different from the other retinal proteins. Three of them should be mentioned: First, the absorption spectrum of pR_{490} has fine structure even at room temperature. Second, P_{520}, corresponding to the K intermediate of the other retinal proteins, has a high thermal stability in comparison with them. Third, a putative intermediate corresponding to L does not appear in the photoreaction cycle of pR_{490}.

The absorption spectrum of pR_{490} has a maximum at 490 nm and a shoulder at 460 nm, which displays a distinguishable peak on cooling to low temperature. A possibility that pR_{490} might be a mixture composed of two or more distinct species is excluded by the fact that the course of bleaching of pR_{490} at room temperature demonstrated identical first-order kinetics at the peak (490 nm) and the shoulder (460 nm) (Figure 2, inset). The similarity in shape between the absorption spectrum of pR_{490} and the action spectrum of phototaxis of bacterium (Takahashi et al., 1988) also indicates that these two peaks are due to fine structure of the single species.

Generally, a fine structure in the absorption spectrum of a retinal protein can be observed when the C_6–C_7 bond of the chromophore is planer or the rotation around the C_6–C_7 bond is suppressed by the protein moiety (Honig et al., 1971). Takahashi et al. suggested that pR_{490} has fine structure in its absorption spectrum caused by the fixation of the C_6–C_7 bond of chromophore (Takahashi et al., 1990). The chromophore binding site of pR_{490} would have a pocket in which the β-ionone ring of the chromophore is tightly held, resulting in a fixed conformation around the C_6–C_7 bond.

Unlike pR_{490}, P_{520} has a broad absorption spectrum without any vibrational structure. The chromophore of P_{520} should be in a 13-cis form considering the fact that the photoisomerization of the chromophore from the all-trans to the 13-cis form is essential for formation of P_{350} (Yan et al., 1990). Therefore, a simple explanation for the disappearance of the fine structure might be due to a distortion of the chromophore by photoisomerization, resulting in a shallow potential surface in its ground state. Although this explanation seems to be acceptable in general, it would not be suitable for P_{520} for the following reasons.

K intermediates of retinal proteins other than pR are converted to the next intermediates above −120 or −140 °C (K, −120 °C; hR_{600}, −120 °C; bovine bathorhodopsin, −140 °C), while P_{520} was stable below −70 °C. It has been generally accepted that in bR or hR, the K intermediate is the primary product of the photoisomerization of the retinylidene chromophore from the all-trans to the 13-cis form without a large conformational change in the protein moiety. The K intermediate has a twisted 13-cis chromophore which is stable only at very low temperature, because the conformational change of the protein moiety which induces relaxation of the twisted chromophore cannot occur at low temperature. Above −120 °C, the thermal conformational change of the protein moiety makes it possible for the chromophore to convert to a relaxed form, resulting in formation of the L intermediate. If the chromophore of P_{520} is as twisted as that of K, it must be converted to the next intermediate near −120 °C, or revert to the original pigment when its conformational change of the protein moiety is inhibited by freezing of the sample (Yoshizawa & Wald, 1967; Imamoto et al., 1989). The fact that P_{520} is stable even at −70 °C suggests that the strain of the chromophore of P_{520} is small enough to keep its structure at this temperature.

Therefore, we will propose another explanation that a twisted structure of the chromophore as the result of the photoisomerization would be relaxed by releasing the fixing of the β-ionone ring region of the chromophore in P_{520}. Thus, the C_6–C_7 bond of the chromophore of P_{520} would be possible to take flexible angles. The isomerization of the *all-trans*- to the 13-*cis*-retinylidene chromophore shortens the longitudinal distance of chromophore (Matsumoto & Yoshizawa, 1978), but the shape of the chromophore binding site would hardly change because of freezing the protein moiety at −170 °C. These facts may indicate that the β-ionone ring moves toward the Schiff base linkage in the chromophore binding site of pR_{490}. This movement would liberate the β-ionone ring from the pocket, resulting in disappearance of the effect of steric hindrance. Since the chromophore binding site of pR_{490} suppresses the rotation around the C_6–C_7 bond, it would interact with the chromophore at two methyl groups of C_1 on the assumption that the C_6–C_7 bond is in a trans form like bR, hR, or sR (Harbison et al., 1985; Baselt et al., 1989).

At −170 °C, P_{480} was formed concurrently with P_{520} by photoreaction. The absorption spectrum of P_{480} was 10-nm blue-shifted from that of pR_{490}. P_{480} was converted to pR_{490} above −160 °C in the dark. The absorption spectrum of P_{480} was very similar in shape to that of pR_{490}, suggesting that the chromophore–protein interaction would be almost identical with that of pR_{490}. Therefore, the chromophore of P_{480} should be in an all-trans form like that of pR_{490}. If P_{480} is formed

from pR_{490}, pR_{490} is photochemically converted to P_{480} without the chromophore isomerization. It seems to be unlikely. On the other hand, in the photoconversion process of pR_{490} to P_{520} with 436-nm light at −170 °C, the intersection points of the spectra shifted to longer wavelengths (Figure 3a). This fact indicates that the red-shifted photoproduct (P_{520}) was formed first and the blue-shifted one (P_{480}) was formed later. These observations lead us to suggest that the precursor of P_{480} is P_{520}. P_{480} would be produced by photoisomerization of the chromophore of P_{520} from the 13-cis to the all-trans form (Figure 10). On the 13-cis to all-trans isomerization, the longitudinal distance of the chromophore is extended, and the β-ionone ring would enter to the pocket again. The steric interaction of the β-ionone ring of P_{480} would be somewhat different from that of pR_{490}, resulting in the thermal conversion of P_{480} to pR_{490} at such a low temperature as −160 °C.

As already described above, P_{520} has high stability and directly converted to P_{350} without formation of any intermediate. This fact is very interesting because not only bR and hR but also animal visual pigments have their own L (lumi) intermediates, which are thought to be the key intermediates to elucidate the conformational change of the protein moiety around the chromophore resulting in the occurrence of chromophore relaxation. As already stated, the chromophore of P_{520} seems to have a small strain, while the shape of the chromophore binding site is similar in shape to that of pR_{490}. On isomerization of pR_{490} from the all-trans to the 13-cis form, the methyl group at C_{13} would displace its position in the chromophore binding site, suggesting that the retinal binding site of pR_{490} has enough space near C_{13} for both the *all-trans*- and the 13-*cis*-retinylidene chromophore and the relaxation of the chromophore in P_{520} did not take place below −60 °C.

P_{350}, showing an absorption maximum in the near-UV region, seems to correspond to the M intermediate of bR. M is the only intermediate whose chromophore is in an unprotonated state in the photoreaction cycle of bR. Therefore, it is reasonable to speculate that the proton attached to the chromophore is released during the conversion process of P_{520} to P_{350}. P_{350} was thermally converted to P_{530}. Because this red-shifted intermediate was converted to pR_{490}, P_{530} seems to correspond to the O intermediate of bR.

pR is a photoreceptive protein responsible for the phototaxis of *H. halobium* in cooperation with sR and S_{373}. Low-temperature spectrophotometry of sR revealed that sR_K was not formed at low temperature (Ariki et al., 1987), while sR_K and sR_L were observed at room temperature by flash photolysis (Ohtani et al., 1986). On the other hand, it was suggested that *H. halobium* has an enzymatic signal transduction system from a photoreceptive protein to flagella (Spudich et al., 1989), which was observed in both pR and sR systems. These results suggest that the mechanism of photoreaction and subsequent thermal reaction of sR are quite different from those of pR, while their physiological functions are very similar.

Acknowledgments

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Registry No. *all-trans*-Retinal, 116-31-4; 13-*cis*-retinal, 472-86-6.

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