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An Amino Acid Residue (S201) in the Retinal Binding Pocket Regulates the Photoreaction Pathway of Phoborhodopsin

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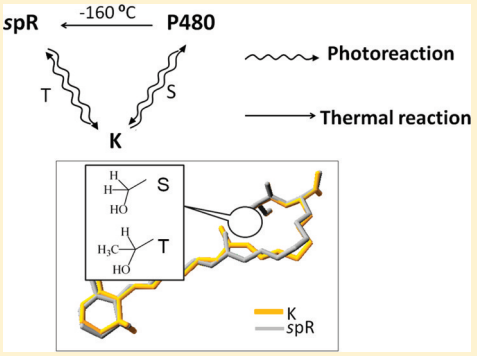
Supporting Information

ABSTRACT: Phoborhodopsin from *Halobacterium salinarum* (*salinarum* phoborhodopsin, *sp*R also called *Hs*SR II) is a photoreceptor for the negative phototaxis of the bacterium. A unique feature of *sp*R is the formation of a shorter wavelength photoproduct, P480, observed at liquid nitrogen temperature beside the K intermediate. Formation of similar photoproduct has not been reported in the other microbial rhodopsins. This photoproduct showed its maximum absorbance wavelength (λ_{max}) at 482 nm and can thermally revert back to *sp*R above −160 °C. It was revealed that P480 is a photoproduct of K intermediate by combination of an irradiation and warming experiment. Fourier transform infrared (FTIR) difference spectrum of P480 from *sp*R in C−C stretching vibration region showed similar features with that of K intermediate, suggesting that P480 has a 13-*cis*-retinal chromophore. The appearance of a broad positive band at 1214 cm^{-1} in the P480-*sp*R spectrum suggested that configuration around C9═C10 likely be different between P480 and K intermediate. Vibrational bands in HOOP region (1035 to 900 cm^{-1}) suggested that the chromophore distortion in K intermediate was largely relaxed in P480. The amount of P480 formed by the irradiation was greatly decreased by amino acid replacement of S201 with T, suggesting S201 was involved in the formation of P480. According to the crystal structure of *pharaonis* phoborhodopsin (*pp*R), a homologue of *sp*R found in *Natronomonas pharaonis*, S201 should locate near the C14 of retinal chromophore. Thus, the interaction between S201 and C14 might be the main factor affecting formation of P480.



Microbial rhodopsins were first found in the membrane of halophilic archaea. Intense studies on the proteins in the past decades made them one of the best understood membrane embedded proteins.[1] Genome sequencing of both cultivated and uncultivated microorganisms since 1999 revealed the broad existence of rhodopsin homologues in the other two domains of life, namely bacteria and eukarya that further broadened our knowledge of the proteins. All microbial rhodopsins that have been isolated from host cell or obtained from heterologous expression system so far have an *all-trans*-retinal as the chromophore, although the visual pigments of higher organisms have 11-*cis* form of retinal chromophore. Light activation of the proteins first causes an isomerization of the chromophore from *all-trans* configuration to 13-*cis* one, triggering a cascade of cyclic photoreactions.

Microbial rhodopsins can be classified into two types according to their putative physiological functions, namely the light-driven ion pumps and photosensors. Phoborhodopsin from *Halobacterium salinarum* (*salinarum* phoborhodopsin, *sp*R also *Hs*SRII) was first reported in 1985 as the fourth retinal pigment residing on the cytoplasmic membrane of *Halobacterium salinarum*.[2] This protein is responsible for the photorepellent response of the bacterium. Absorption maximum of this pigment was at 490 nm with a shoulder due to the vibrational fine structure at 460 nm.[3,4] Photoreaction of *sp*R was studied by flash photolysis[5] and by low-temperature spectroscopy.[4,6] Formation of K, M, and O intermediate was observed. In addition, formation of a new photoproduct, P480, was observed by irradiation of *sp*R at −170 °C, which reverted thermally to *sp*R at the temperature above −160 °C. In our previous work, the photoreaction cycle of *E. coli*-expressed *sp*R was studied by low-temperature spectroscopy.[7] Formation of P480 and K intermediate at −180 °C was confirmed.

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Comparing the photochemical properties of *sp*R with that of *pharaonis* phoborhodopsin (*pp*R), a homologue of *sp*R found in *Natronomonas pharaonis*, and that of bacteriorhodopsin (BR), there are several differences between them: (i) the absorption maximum of *sp*R is 486 nm, which is about 10 nm shorter than that of *pp*R (497 nm) and more than 80 nm shorter than that of BR (568 nm); (ii) formation of a shorter wavelength photoproduct, P480, beside the K intermediate was observed in *sp*R; (iii) M and O intermediates appeared to be more thermally stable than those of *pp*R and BR.

Formation of P480 at liquid nitrogen temperature is a unique character of *sp*R, since the similar photoproduct has not been observed in the other microbial rhodopsins. This photoproduct shows maximum absorbance at 482 nm and can thermally revert back to *sp*R above −160 °C. In the case of BR and *pp*R, photoexcitation of the initial state selectively isomerizes the C13=C14 double bond of the retinal chromophore from the *trans* configuration to the *cis* one. A longer wavelength photoproduct, K intermediate, is produced during this process. On the other hand, light excitation of the K intermediate converts the photoproduct into its initial state without producing any side product like P480. Formation of a photoproduct (P480) beside K intermediate in *sp*R suggests that there is a unique protein−chromophore interaction in *sp*R. Study on P480 might shed a light on the understanding of the chromophore−protein interaction that regulates the primary photoreaction of the retinal proteins, such as visual pigment rhodopsins and microbial rhodopsins.

In the present work, we studied P480 in more detail. We aimed to elucidate the interaction between the retinal chromophore and the amino acid in the retinal binding pocket that is responsible for the formation of P480. By low-temperature spectroscopic studies, it was elucidated that P480 is a photoproduct of K intermediate. Fourier transform infrared (FTIR) spectroscopic study of P480 suggested that the chromophore configuration of P480 is likely to be in 13-*cis* 15 anti form. Comparing the FTIR difference spectrum of P480 with that of K intermediate of *sp*R, the large difference between them was found in the HOOP region. The retinal torsion observed in K intermediate was largely relaxed in P480. Spectroscopic studies on the *sp*R samples with amino acid replacement indicated that the formation of P480 might be resulted from the different chromophore environment around the C14 of retinal chromophore in *sp*R.

MATERIALS AND METHODS

Preparation of *sp*R and Its Mutants. The *sp*R-expression plasmid (pET21c/*sp*RHis) was constructed as previously described.[7] The plasmid for *sp*R-D103N, *sp*R-Y132F, and *sp*R-S201T mutants was prepared by PCR with the QuikChange site-directed mutagenesis kit (Stratagene). The primers (5′-CTGCTCGC CGCCAACGTGTTCGTCAT C-3′ and 5′-GATGACGAACACGTTGGCGGCGAGCAG-3′ for *sp*R-D103N, 5′-CTGCCGGCTTCGCCGCGCTCCTGTAC-3′ and 5′-GTACAGGAGCGCGGCGAAGCCGGCAG-3′ for *sp*R-Y132F, 5′-CTGGATTTCATCACGAAGGTCGCGTTC-3′ and 5′-GAACGCGACCTTCGTGATGAAATCCAG-3′ for *sp*R-S201T) were designed to introduce each substitution. Underlined bases indicate the substituted bases. 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.*[8] *E. coli* BL21-CodonPlus (DE3)-RP cells (Stratagene) were transformed by pET21c/*sp*RHis. The cells were grown in 2 × YT medium supplemented by ampicillin (final concentration of 50 μg/mL) to OD660 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 (4700*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 (3300*g*, 10 min, 4 °C) and resuspended in buffer A, followed by sonication on ice. The cell debris were removed by centrifugation (3300*g*, 3 min, 4 °C), and membrane fraction was collected by ultracentrifugation (106000*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 ultracentrifugation (106000*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 nonspecific materials were removed. Protein (*sp*R) was eluted by Buffer E (4 M NaCl, 250 mM imidazole, 50 mM MES, pH 6.0, and 0.05% DDM). Three mutant proteins were further purified by gel filtration column chromatography. Finally, the medium was exchanged into the solution (4 M NaCl plus 0.05% DDM buffered with 7-mixed buffer (containing citrate/MES/HEPES/MOPS/Tris/CHES/CAPS, the final concentration of each component was 10 mM) at pH 5.0) by Sephadex G-25 (GE Healthcare, Tokyo), and the sample was concentrated by ultrafiltration (Amicon Ultra-15, Millipore). For FTIR measurements, proteins were reconstituted into L-α-phosphatidylcholine (PC) from egg yolk (Avanti, Alabaster, AL) at an protein:PC molar ratio of 1:50 by removing detergent by gentle stirring overnight at room temperature in the presence of SM2 Adsorbent Bio-Beads (Bio-Rad, Hercules, CA). During the preparation of the pigment, we tried not to expose the sample under light as much as possible.

Low-Temperature Spectrophotometry. For measurements of the absorption spectra at low temperature, the purified *sp*R or its mutants in 4 M NaCl, 10 mM 7-mixed buffer, pH 5.0, and 0.05% DDM was suspended with glycerol in a final concentration of 66%. Absorption spectra were measured by a MPS 2000 recording spectrophotometer (Shimadzu) equipped with a glass cryostat as described previously.[9] The temperature of the sample was monitored with a copper−constantan thermocouple attached to the cell holder. The sample was irradiated with the light from a 300 W halogen projector lamp passed through an interference filter and/or cutoff filter. In order to decrease heat of the light, the irradiation light was passed through a 5 cm water layer. The intensity of the irradiation light with the interference filter (450 nm) and a cutoff filter (>540 nm) was 20 μW at the position of the sample holder.

FTIR Spectroscopy. FTIR spectroscopy was applied as described previously.[10] Eighty μL of each *sp*R sample in 2 mM phosphate buffer (pH 5.0) was dried on a BaF_2 window with a diameter of 18 mm. After hydration by either H_2O or D_2O, the sample was placed in a cell, mounted in an Oxford DN-1704 cryostat, and cooled to −180 °C.

For the measurement of difference spectra, *sp*R sample was cooled down to −180 °C (A state) and irradiated with 450 nm light for 2 min (B state) followed by irradiation with >540 nm light for 2 min (C state). After recording the spectra of A, B, and C states, the photoproducts mixture was heating up to −130 °C followed by cooling down to −180 °C to repeat the same measurements. An irradiation with 450 nm light produces a mixture of *sp*R, P480, and K intermediate and an irradiation with >540 nm light produces a mixture of *sp*R and P480.[7] P480 was reverted to *sp*R by heating up to −130 °C. The difference spectrum between P480 and *sp*R was obtained by subtracting the spectrum of A state from C state and the K-*sp*R difference spectra were calculated as $B - A + 0.25 \times (C - A)/0.45$. The calculation method of the spectra is described in the Supporting Information. The difference spectra were calculated from the spectra constructed with 128 interferograms, and 24 spectra obtained in this way were averaged.

RESULTS AND DISCUSSION

P480 Is a Photoproduct of K Intermediate. At 20 °C, *sp*R showed its maximum absorbance at 486 nm (curve 1 in Figure 1a). After cooling down to −180 °C, the maximum absorbance wavelength (λ_{max}) did not change, but the peaks became sharper and higher. Prolonged irradiation of *sp*R with 450 nm light at −180 °C produces a photo-steady state containing 51.8% of a longer wavelength photoproduct, K intermediate, and 25% of a shorter wavelength photoproduct, P480 (see Supporting Information). Under the conditions of our irradiation system mounted with an interference filter (450 nm), the time for producing 50% of the maximum amount of K was about 10 s (Figure 1b). P480 thermally reverts back to *sp*R above −160 °C, and the K intermediate converts to L intermediate above −80 °C.[4,7]

To investigate the precursor of P480, we compared the amounts of P480 in following states: (1) State I, photoproducts mixture (curve 2 in Figure 2a) produced by irradiating *sp*R (curve 1 in Figure 2a) with 450 nm light for 4 min at −180 °C. This photoproducts mixture contains *sp*R, P480, and K intermediate. (2) State II, photoproducts mixture (curve 3 in Figure 2a) produced by further irradiation of state I (curve 2 in Figure 2a) with >580 nm light for 10 min at −180 °C. Only K intermediate absorbs >580 nm light and undergoes photoconversion upon

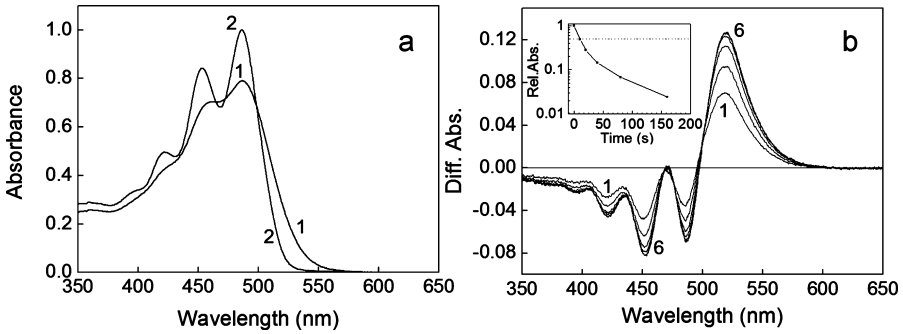

Figure 1. Absorption spectrum and photoreaction of *sp*R. (a) Absorption spectrum of *sp*R. Curve 1 is the absorption spectrum measured at 20 °C, and curve 2 is that at −180 °C. The samples were suspended in a medium containing 4 M NaCl, 50 mM MES, pH 5.0, and 0.05% DDM. For the low-temperature spectroscopy measurement, 66% glycerol was added into this medium. (b) Difference spectral changes before and after the irradiation of *sp*R with 450 nm light for a total period of 10, 20, 40, 80, 160, and 320 s (curves 1−6, respectively). The absorption spectrum of *sp*R at −180 °C was recorded as a baseline. As shown in these curves, a photo-steady state (curves 5 and 6) was finally attained. Inset shows the kinetics of absorbance change at 486 nm $((A - A_\infty)/(A_0 - A_\infty))$.

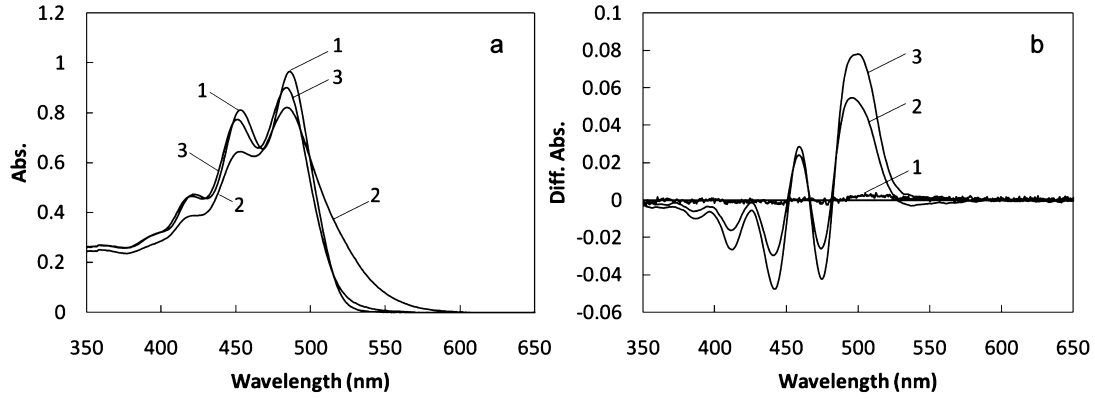

Figure 2. P480 is a photoproduct of K intermediate. (a) Photoreaction of *sp*R at −180 °C. Curve 1, *sp*R. Curve 2, state I, obtained by irradiating *sp*R with 450 nm light for 4 min. Curve 3, state II, photoproduct mixture obtained by irradiating *sp*R with 450 nm light for 4 min followed by irradiation with >580 nm light for 10 min. (b) Curve 1, difference spectrum before and after the irradiation of *sp*R (curve 1 in panel a) with >580 nm light for 10 min at −180 °C. Curve 2, difference spectrum between the state I (curve 2 in panel a) and the product after the state I was heating up to −150 °C and kept for 5 min to convert all P480 into *sp*R followed by recooling down to −180 °C. Curve 3, difference spectrum between the state II (curve 3 in panel a) and the product after the state II (curve 3 in panel a) was heating up to −150 °C and kept for 5 min to convert all P480 into *sp*R followed by recooling down to −180 °C.

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irradiation (see curves 1 and 3 in Figure 2a). This photoproduct mixture contains *s*pR, P480, and remaining K intermediate. Curves 2 and 3 in Figure 2b show the difference spectral changes before and after the two states were heated up to −150 °C and kept in the dark for 5 min before cooling down to −180 °C to measure the spectra. Since only P480 converted back to *s*pR at −150 °C, the amplitude of difference spectra represents the relative amounts of P480 in the two states. Amplitude of the peaks in curve 2 is smaller than that in curve 3, suggesting that amount of P480 in state I (curve 2 in Figure 2a) was smaller than that of state II (curve 3 in Figure 2a). The additional amount of P480 in state II (curve 3 in Figure 2a) should be produced from the photoconversion of K intermediate since the irradiation of *s*pR with >580 nm light for 10 min caused little change in the absorption spectrum (curve 1 in Figure 2b). Therefore, it can be concluded that P480 is a photoproduct of K intermediate.

The fact that irradiation of *s*pR with blue light can produce a photo-steady state containing *s*pR, K, and P480 suggests that P480 should be also photoactive like *s*pR and K intermediate; otherwise, P480 would accumulate continuously with the irradiation, and finally all *s*pR and K should convert to P480. Because our results showed that P480 is a photoproduct of K intermediate, the photoproduct of P480 is most likely to be K intermediate. Hence, the photoreaction scheme at −180 °C would be like the following:

$$s\text{pR} \underset{\phi_{-1}}{\overset{\phi_1}{\rightleftharpoons}} \text{K} \underset{\phi_{-2}}{\overset{\phi_2}{\rightleftharpoons}} \text{P480}$$

The ratio of quantum yield of *s*pR-to-K transition and its back-reaction (ϕ_1/ϕ_{-1}) was calculated to be 1.38, which is much higher than that of *p*pR-to-K transition and its back-reaction (0.70[11]). If the quantum yield of the forward reaction of *s*pR (ϕ_1) is similar to that of *p*pR, the quantum yield of K-to-*s*pR transition (ϕ_{-1}) is much smaller than that of *p*pR. The ratio of quantum yield of P480-to-K transition and its back-reaction (ϕ_{-2}/ϕ_2) was calculated to be 1.29.

Chromophore Structure of P480 Studied by FTIR Measurement. In order to clarify the chromophore configuration of P480, the FTIR difference spectra of P480 and K intermediates were measured. Figure 3 shows the C−C stretching vibrations of the retinal chromophore in the 1300−1100 cm^{-1} region, which is sensitive to the chromophore configuration. As shown in Figure 3a, negative bands at 1255, 1244, 1226, and 1203 cm^{-1} and positive bands at 1195 and 1188 cm^{-1} were observed in the *s*pR_K-*s*pR spectrum. The P480-*s*pR spectrum (Figure 3b) showed a similar feature with that of *s*pR_K-*s*pR, except the appearance of bands at 1214 and 1184 cm^{-1} and the disappearance of the band at 1188 cm^{-1}. These two spectra were compared with BR_K-BR spectrum (Figure 3c).[12] The bands in the BR_K-BR spectrum have been studied extensively and assigned as follows. The negative bands at 1255, 1216, 1203, and 1169 cm^{-1} in Figure 3c were assigned to the C−C stretching vibrations of the retinal chromophore at positions C12−C13, C8−C9, C14−C15, and C10−C11, respectively.[13,14] The negative 1255 cm^{-1} band is composed of a mixture of D_2O-insensitive C12−C13 stretching and D_2O-sensitive N−H in-plane bending vibrations.[15] A positive 1194 cm^{-1} band originates from C14−C15 and C10−C11 stretches.[16] From the similarity in frequency, we tentatively assigned the negative bands at 1255, 1244, and 1203 cm^{-1} in the *s*pR_K-*s*pR spectrum as the C12−C13 stretch, N−H bend, and C14−C15 stretches in *s*pR. The positive band at

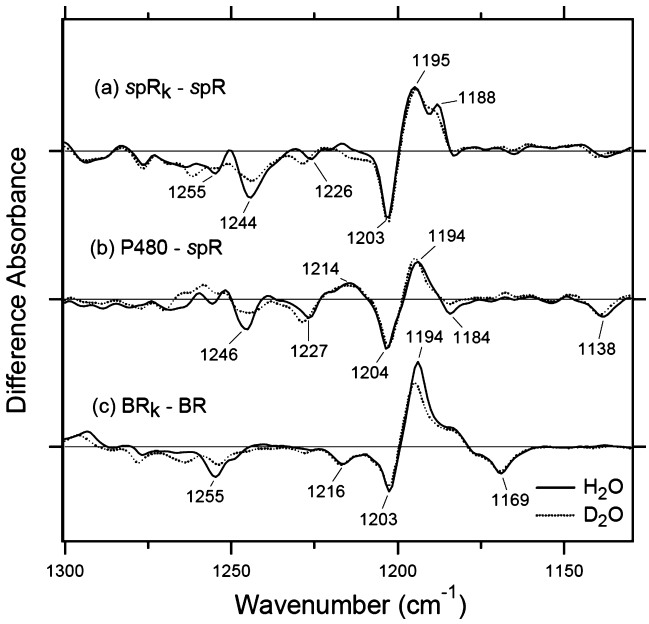

Figure 3. Difference FTIR spectra in the 1300−1100 cm^{-1} region: (a) *s*pR_K minus *s*pR spectra; (b) P480 minus *s*pR spectra; (c) BR_K minus BR spectra. The sample was hydrated with either H_2O (solid lines) or D_2O (dotted lines).

1195 cm^{-1} would originate from C14−C15 stretches. The positive band at 1188 cm^{-1}, however, is somewhat difficult to assign. It seems like the C10−C11 stretch of K intermediate; however, the lack of a negative band around 1169 cm^{-1} indicated there was no changes in C10−C11 upon formation of K. The negative band at 1227 cm^{-1} may originate from protein vibrations because of its absence in the resonance Raman spectra of *s*pR.[8] Similarly, the negative bands at 1246 and 1204 cm^{-1} in the P480-*s*pR spectrum are assignable to the N−H bend and C14−C15 stretches, respectively, and the positive band at 1195 cm^{-1} to C14−C15 stretches. The negative band at 1227 cm^{-1} may originate from protein vibrations.

A broad positive band at 1214 cm^{-1} was observed only in P480-*s*pR spectrum. Interestingly, a similar band was observed in pink membrane which has 9-*cis*-retinal chromophore[17] and in 9-*cis* species of proteorhodopsin.[18] It was suggested that this band originated from the C8−C9 stretch coupled to the C9−CH_3 stretch and C10−H in-plane rock.[17] If the band at 1214 cm^{-1} is assignable to C8−C9 stretch, appearance of the band at this position suggested that configuration around C9═C10 was different between P480 and K intermediate.

The appearance of a negative band at about 1203 cm^{-1} and a positive band at about 1194 cm^{-1} has been considered to be one of the characters of the formation of K intermediate in microbial rhodopsins (the wavenumber in the case of BR). The negative and positive bands originate from the stretching vibration of C14−C15 in BR initial state and K intermediate, respectively. The downshift would result from the geometric changes of the retinal molecule caused by isomerization of C13═C14, which leads to different coupling of the C14−C15 stretching vibration with bending vibrations. Interestingly, similar bands were also observed in P480 minus *s*pR spectra (Figure 3b), suggesting that P480 might also have a 13-*cis*-retinal as the chromophore. To confirm it, the C14−C15 bands of *s*pR_K-*s*pR and P480-*s*pR were quantitatively compared by

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normalizing the amount of K and P480 as unity. Amplitudes of C14−C15 bands were close in two difference spectra (data not shown), suggesting that the chromophore configuration of P480 should be 13-*cis*.

The FTIR difference spectra in the 1300−1100 cm^{-1} region (Figure 3) also provide us the information about the configuration of C=N. The coupling of C14−Cl5 stretch with C15NH rock is drastically different for C=N *cis* (15 syn) and C=N *trans* (15 anti) molecules.[13] In the 15 syn configuration the rock-stretch coupling is large, deuteration of the Schiff base nitrogen results in a 40−60 cm^{-1} shift of the C14−C15 stretch. However, in the 15 anti configuration, the rock-stretch coupling is small; deuteration of the Schiff base nitrogen produces only a slight (<5 cm^{-1}) shift of the C14−C15. In BR the light adapted form has *all-trans* 15 anti chromophore and the dark-adapted form has both *all-trans* 15 anti and 13-*cis* 15 syn chromophore. K intermediate of light adapted BR has 13-*cis* 15 anti chromophore.[19] As shown in Figure 3a,b, the C14−C15 stretch of *s*pR (negative band at 1203 or at 1204 cm^{-1}), K intermediate (positive band at 1195 cm^{-1}), and P480 (positive band at 1194 cm^{-1}) were insensitive to the deuteration of the Schiff base nitrogen, suggesting the C=N configuration in all three states was 15 anti.

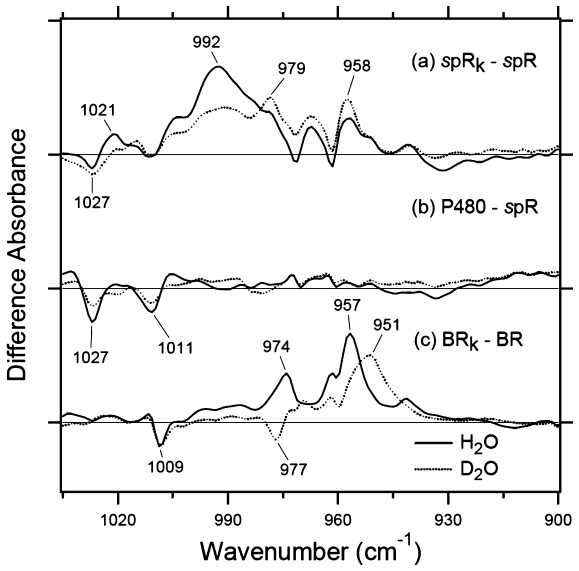

Figure 4. Difference FTIR spectra in the 1035 −900 cm^{-1} region: (a) *s*pR$_K$ minus *s*pR spectra; (b) P480 minus *s*pR spectra; (c) BR$_K$ minus BR spectra. The sample was hydrated with either H_2O (solid lines) or D_2O (dotted lines).

Figure 4 compares hydrogen out-of-plane (HOOP) vibrations of the retinal chromophore which provides the information of chromophore distortion. Appearance of intense positive bands in the primary photoproduct of retinal proteins in this region has been interpreted as the presence of chromophore distortion in the primary photoproduct. In *s*pR$_K$-*s*pR spectra (Figure 4a) intense positive D_2O-sensitive band at 992 cm^{-1} and D_2O-insensitive bands at 968 and 958 cm^{-1} were observed. In contrast, the spectral changes in P480 in this region (Figure 4b) were very small, except the negative bands at 1011 and 1027 cm^{-1}. In BR$_K$-BR spectra (Figure 4c) D_2O-sensitive intense bands at 974 and 957 cm^{-1} were assigned as HOOP vibrations of C15−H and N−H groups,[15] and the D_2O/H_2O exchange-insensitive negative peak at 1009 cm^{-1} was assigned as the symmetric in-plane methyl rocking combination involving mainly the methyl groups at C9 and C13 positions in BR.[20] These spectral changes were interpreted as the retinal distortion upon BR$_K$ formation is localized in the Schiff base region. This interpretation was also supported by the crystallographic study of K intermediate of BR.[19] Appearance of intense D_2O-sensitive and D_2O-insensitive bands in the *s*pR$_K$-*s*pR spectrum indicates that the chromophore distortion is more extended. It should be noted that this spectral feature is very similar with that of the *p*pR. In the *p*pR$_K$-*p*pR spectrum, the D_2O-insensitive bands at 966 and 958 cm^{-1} were assigned as the C7=C8 and C11=C12 Au (irreducible representation of diene vibrations) HOOP modes, respectively, and the D_2O-sensitive positive bands at 1001, 994, 987, and 979 cm^{-1} as the C15-HOOP vibrations.[21] From the similarity in frequency, the bands at 968 and 958 cm^{-1} were assigned as C7=C8 and C11=C12 Au HOOP modes, respectively, and the band at 992 cm^{-1} was assigned as C15-HOOP vibrations. Thus the chromophore distortion in K intermediate of *s*pR is reached to C7 of retinal like that of *p*pR. However, in P480 the chromophore distortion is largely relaxed and became almost the same with that of *s*pR. Note the negative band at 1011 cm^{-1} is attributable to the in-plane vibration of two methyl groups in the C9 and C13 positions, and the 1027 cm^{-1} band might be a protein band, since resonance Raman spectroscopy did not show any peak at this position.[8] Since both BR and *p*pR have a relatively planar chromophore in their ground states, it is reasonable to conceive that *s*pR also has a relatively planar chromophore in its ground state. It is also supported by the lack of an intense band in HOOP region of *s*pR in resonance Raman spectroscopy[8] and prominent vibrational fine structure in the absorption spectrum of *s*pR.[3] Little change in P480-*s*pR in HOOP region suggests that P480 would have a relatively planar chromophore as ground state *s*pR.

In summary, the K intermedaite of *s*pR has a distorted 13-*cis* 15 anti chromophore and P480 has a relatively planner 13-*cis* 15 anti chromophore. Configuration around C9=C10 is likely different between P480 and K intermediate. One possibility is that P480 has a 9,13-di-*cis*-retinal chromophore.

Formation of P480 Was Mostly Affected by the Amino Acid Replacement near the Retinal Schiff Base. In order to identify the key residue responsible for the formation of P480, we replaced three amino acids in the retinal binding pocket of *s*pR with the corresponding ones of *p*pR. The three amino acid residues of *s*pR are positioned near the β-ionone ring (Y132 replaced with F), middle of polyene chain (D103 replaced with N), and near the retinal Schiff base (S201 replaced with T). The replacement of these three amino acids did not result in the change of their λ_{max} as shown in Table 1. To produce P480, the three mutants were irradiated with 450 nm light followed by irradiation with >540 nm light. Each irradiation was prolonged until the photoreactions reached to the photo-steady state. Figure 5 shows the difference spectra of P480 and *s*pR or its mutants. The amplitudes of difference minimum represent the relative amount of P480 in the photo-steady states. As shown in Figure 5 and Table 1, although formation of P480 was observed in three mutants, S201 to T replacement has the largest effect on the formation of P480. The amount of P480 formed in *s*pR-S201T mutant was

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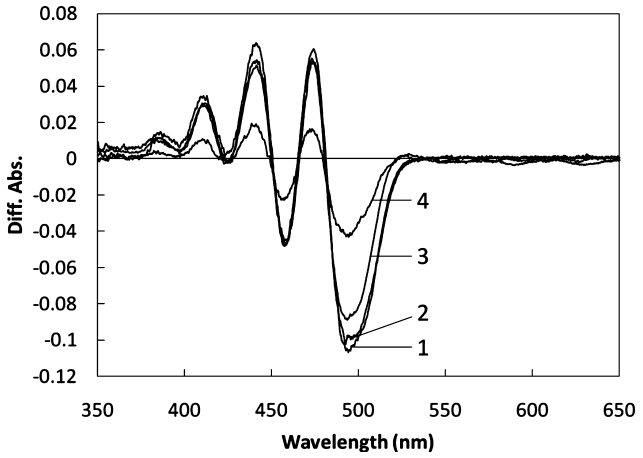

Figure 5. Difference spectra between P480 and *sp*R or its mutants: curve 1, *sp*R wild type (WT); curve 2, Y132F mutant; curve 3, D103N mutant; curve 4, S201T mutant. The difference spectra were obtained by subtracting the absorption spectra of *sp*R or its mutants from that of the photoproduct mixtures containing P480. The photoproduct mixture was obtained by irradiating *sp*R or its mutants with 450 nm light followed by irradiation with >540 nm light at −180 °C. Each irradiation was prolonged to reach a photo-steady state. The absorption spectra of *sp*R and its mutants were normalized at absorption maximum before calculating the difference spectra.

Table 1. Maximum Absorbance Wavelengths (λ_{max}) and Formation of P480 in *sp*R and Its Mutants

	*sp*R WT	*sp*R-Y132F	*sp*R-D103N	*sp*R-S201T
λ_{max} (nm)	485.6	485.4	484.4	485.8
difference minima of the curves in Figure 5	−0.1073	−0.0995	−0.0894	−0.0421
relative amount of P480	100	92.7	83.3	39.2

decreased to 39.2% of that in wild type, suggesting S201 is most likely involved in the formation of P480.

In *pp*R, the corresponding amino acid of S201 of *sp*R is T204, which is important for the function of the protein because the cells containing mutated *pp*R (T204S) did not exhibit phototaxis.[22] The negative phototaxis of *Halobacterium salinarum* under the blue green light suggested that *sp*R with a serine residue in the corresponding position has the physiological function as *pp*R. That indicates the local environment around the residue should be different between *sp*R and *pp*R. Crystallographic study of *pp*R suggested that T204 of *pp*R is near the C14 of retinal.[23] Side chain of T204 has steric interaction with hydrogen of C14 in K intermediate.[24] Thus, it is reasonable to conceive the difference in local environment around C14 of retinal chromophore might be responsible for the formation of P480 in *sp*R.

The corresponding residue of S201 of *sp*R is alanine in BR. Alanine residue at this position is conserved in all proton pumps, chloride pumps, and sensory rhodopsin I of haloarchaea.[25] In phoborhodopsins this residue is replaced with the polar unchargeable residues such as serine in *sp*R or threonine in *pp*R. Among the three amino acids, the hydrophobicity of alanine is highest with the hydropathy index of 1.8. The hydrophobicity of threonine (hydropathy index −0.7) is higher than that of serine (hydropathy index −0.8) because of an additional methyl group. Our results showed that hydrophilic residue near the C14 of retinal prefers the formation of P480 and hydrophobic one prefers the back reaction to original pigment (Figure 6).

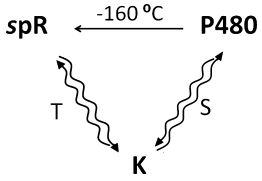

Figure 6. Photoreaction of *sp*R at −180 °C. Wavy lines represent the photoreactions, and straight line represents the thermal reaction. Serine residue in the 201 position prefers the formation of P480 while threonine residue prefers the back reaction to *sp*R .

Although formation of P480 was greatly depleted in S201T mutant of *sp*R, amino acid replacement of S201 with T did not eliminate the formation of P480 completely. That suggests there should be other factors that contribute to the formation of P480. It should be noted that the negative band around 1009 cm^{-1} in the BR_K-BR spectrum was not observed in FTIR spectra of spR_K-*sp*R. A similar negative band was also observed in the ppR_K-*pp*R spectrum at 1013 cm^{-1}.[12] This band was assigned as the symmetric in-plane methyl rocking combination involving mainly the methyl groups at C9 and C13 positions. Lack of the negative band around 1009 cm^{-1} in the spR_K-*sp*R spectrum suggests that the environment around methyl groups remains unchanged upon formation of K intermediate. Thus, it is possible that the different environment around the methyl groups of C13 and C9 might also contribute to the formation of P480.

CONCLUDING REMARKS

In the present work we studied the photoproduct, P480, formed at −180 °C in detail. Low-temperature spectroscopic study showed that P480 is a photoproduct of K intermediate. FTIR measurement showed that P480 has a 13-*cis* chromophore with a relaxed chromophore distortion and probable difference in the configuration of C9═C10 double bond with that of the K intermediate. We found that interaction between S201 and C14 might be the main factor affecting the formation of P480. All microbial rhodopsins except for *pp*R and *sp*R have alanine at corresponding position, suggesting that the amino acid residue at this site may be important for the controlling the photoreaction pathway.

ASSOCIATED CONTENT

Supporting Information

Estimation of the amounts of P480 and K intermediate in the photo-steady states. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

*sp*R, phoborhodopsin from *Halobacterium salinarum*; *Hs*SRII, sensory rhodopsin II from *Halobacterium salinarum*; λ_{max}, maximum absorbance wavelength; FTIR, Fourier transform infrared spectroscopy; *pp*R, phoborhodopsin from *Natronomonas pharaonis*; IPTG, isopropyl-1-thio-*β*-galactoside; DDM, *n*-dodecyl-*β*-D-maltoside; HOOP, hydrogen out-of-plane vibration; BR, bacteriorhodopsin.

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