Photoreceptor Proteins

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Color-Discriminating Retinal Configurations of Sensory Rhodopsin I by Photo-Irradiation Solid-State NMR Spectroscopy**

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Abstract: *SRI (sensory rhodopsin I) can discriminate multiple colors for the attractant and repellent phototaxis. Studies aimed at revealing the color-dependent mechanism show that SRI is a challenging system not only in photobiology but also in photochemistry. During the photoreaction of SRI, an M-intermediate (attractant) transforms into a P-intermediate (repellent) by absorbing blue light. Consequently, SRI then cycles back to the G-state. The photoreactions were monitored with the ^{13}C NMR signals of [20-^{13}C]retnal-SrSRI using in situ photo-irradiation solid-state NMR spectroscopy. The M-intermediate was trapped at −40 °C by illumination at 520 nm. It was transformed into the P-intermediate by subsequent illumination at 365 nm. These results reveal that the G-state could be directly transformed to the P-intermediate by illumination at 365 nm. Thus, the stationary trapped M- and P-intermediates are responsible for positive and negative phototaxis, respectively.*

Sensory rhodopsin I (SRI) is a membrane-embedded microbial rhodopsin and functions as a color-discriminating photoreceptor protein in the halobacterial phototaxis.[1] SRI has 7-transmembrane α-helices and in the dark state, has an *all-trans* retinal chromophore.[2,3] SRI forms a 2:2 complex with its cognate transducer protein, halobacterial transducer protein for SRI, HtrI.[4] The essential photocycle of SRI from *Halobacterium salinarum* (*Hs*SRI) proceeds as follows: $SRI_G(587)\rightarrow(h\nu)\rightarrow SRI_K(620)\rightarrow SRI_L(540)\rightarrow SRI_M(373)\rightarrow SRI_G(587)$. Additionally, the $SRI_P(520)$ intermediate is produced from the M-intermediate by a second photon absorption of near-UV light as a back reaction to SRI_G; that is, $SRI_M(373)\rightarrow(h\nu)\rightarrow SRI_P(520)\rightarrow SRI_G(587)$.[5–7] Here, SRI_P is the same intermediate as S^b, which was previously characterized by flash-induced absorbance changes of SRI_M.[1,5] During the photocycle, the M- and P-intermediates are thought to be essential for the positive and negative phototaxis, respectively (Supporting Information, Figure S1).

One-photon reaction (red light) generates an attractant signal (M-intermediate), which suppresses flagellar reversal as compared to the non-signaling state (G-state). In contrast, a two-photon reaction (red + blue lights) generates a repellent signal (P-intermediate), which induces flagellar reversals as compared to the non-signaling state (G-state).[1]

Thus, the structural changes of both the protein moiety and the retinal chromophore upon formation of the active M- and P-intermediates continue to be an exciting topic of discussion, although high-resolution structural studies have been a major challenge. Further, solid-state NMR techniques can be applied to elucidate the conformations of photoactivated intermediates and the photocycle pathway with atomic resolution of such membrane-embedded systems under natural conditions.

However, the inherent instability of *Hs*SRI hampers the elucidation of its molecular mechanism. Recently, a novel SRI protein from a eubacterium *Salinibacter ruber* (*Sr*SRI) was cloned and characterized. This is the first eubacterial SRI identified as a functional protein.[8] In the dark state, *Sr*SRI has an *all-trans* retinal as a chromophore, has an absorption maximum at 557 nm, and has a slower photocycle than the light-driven ion pumping rhodopsin (BR and HR), indicating similarities with *Hs*SRI.[8,9] *Sr*SRI is much more stable than *Hs*SRI, especially both in dilute salt solutions and in detergent micelles. These characteristics of *Sr*SRI will allow new approaches to investigate the photo-signaling process of SRI.

In situ photoirradiation solid-state NMR spectroscopy has been developed and used to successfully identify the multiple M-intermediates of *pharaonis* phoborhodopsin (*p*pR or sensory rhodopsin II), and those of the complex with transducer (*p*pR/*p*HtrII) embedded in a model membrane.[10] These intermediates are difficult to distinguish using visible spectroscopy. The ^{13}C NMR signals from [20-^{13}C]retinal-*p*pR and *p*pR/*p*HtrII revealed that multiple M-intermediates with 13-*cis*, 15-*anti* retinal configurations coexist under continuously photoirradiated conditions.[10] It has been reported that the retinal chromophore ^{13}C-labeled at position 20 shows a significant chemical shift from about 14 to 22 ppm upon *trans–cis* photoisomerization. This is in contrast to other

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positions, such as positions 10, 11, and 19[11] (Supporting Information, Table S1). This large and specific shift is also observed in the protein moiety of bacteriorhodopsin[11–13] and sensory rhodopsin II.[10] It was also realized that the ^{13}C NMR intensity of a methyl carbon such as at position 20 is much higher than an olefinic carbon such as at position 15 (data not shown). Thus the retinal chromophore labeling with ^{13}C at position 20 provides a good reporter for investigating retinal configurations.

Herein, we successfully observed the individual intermediates of the photocycle of *Sr*SRI using in situ photo-irradiation solid-state NMR spectroscopy,[10] and obtained the ^{13}C NMR signals from the [20-^{13}C]retinal of long lived M- and P-intermediates. We also investigated the pathways from the G-state to various photo-intermediates to gain insight into the photocycle mechanism of *Sr*SRI.

The significance of our findings is that the cells are able to escape from deleterious UV light by absorbing a second short wavelength photon to form the repellant P-intermediate by reprotonating the Schiff base of *Sr*SRI.

First, ^{13}C solid-state NMR spectra of [20-^{13}C]retinal-*Sr*SRI in the G-state were observed under dark conditions at −40 °C. Subsequently, in situ photo-irradiated ^{13}C solid-state NMR spectra were observed under irradiation with 520 or 595 nm LED light at −40 °C to investigate the photo-isomerization process from the G-state to the M-intermediate (Figure 1).

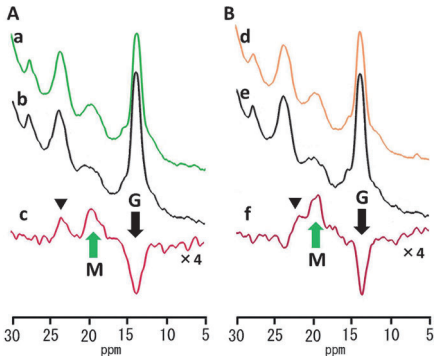

Figure 1. Transformation process from the G-state to the M-intermediate. The ^{13}C NMR signals of [20-^{13}C]retinal-*Sr*SRI-PG system observed in the dark (black; A, spectrum b, and B, spectrum e) and under illumination with 520 nm (green; A, spectrum a) and 595 nm (orange; B, spectrum d) LED light and the difference spectra between the light and dark states (pink, a minus b; A, spectrum c and d minus e; B, spectrum f).

Separate illumination each of two frequencies was performed to clarify which intermediate was the actual M-intermediate. This was necessary as the M-intermediate could be induced by both frequencies, as the absorption maximum of the G-state is 558 nm. Figure 1 A, spectrum b shows the ^{13}C NMR peak of [20-^{13}C]retinal-SRI in the G-state appeared at 13.8 ppm (Supporting Information, Table S1). This peak overlaps with the signals of lipid methyl groups. Figure 1 A, spectrum a shows the ^{13}C NMR spectrum measured under photo-irradiation with 520 nm LED light. Similarly, Figure 1 B, spectrum e shows the ^{13}C NMR spectrum of [20-^{13}C]retinal-SRI in the G-state under dark conditions. Figure 1 B, spectrum d shows the ^{13}C NMR spectrum obtained under illumination with 595 nm LED light. The signals at 13.8 ppm decreased while the signal at 19.8 ppm increased. It is possible to trap the M-intermediate under illumination with 520 or 595 nm LED lights because the half-life of the M-intermediate is the longest of any of the other intermediates.[5] The retinal configuration was assigned based on comparison of the ^{13}C chemical shifts with other microbial rhodopsins for which more detailed data are available as shown in the Supporting Information, Table S1. Thus, the signal at 19.8 ppm could be assigned to the M-intermediate by further comparing its chemical shifts with those of the M-intermediates in *pp*R[10] and BR[11–13] (Supporting Information, Table S1).

As signals from lipids heavily overlapped those from the protein, difference spectra were obtained to clarify the light-induced changes in *Sr*SRI. The difference spectrum of the dark and light states clearly indicated that the ^{13}C NMR signal of the G-state at 13.8 ppm decreased and that of the M-intermediate at 19.8 ppm increased (Figure 1 A, spectrum c and 1 B, spectrum f). This result indicated that the G-state with an *all-trans* retinal[6,14] transformed to the M-intermediate with a 13-*cis*, 15-*anti* configuration of retinal bound through a deprotonated Schiff base (SB). These configurations of retinal in the M-intermediate of *Sr*SRI could be clearly determined by comparing with those of *pp*R[10] and BR[11–13] (Supporting Information, Table S1). Furthermore, the absorption maximum at 390 nm[8] indicated a deprotonated SB. Similar results were obtained by irradiation with 520 nm LED light (Figure 1 A) and 595 nm LED light (Figure 1 B). Interestingly, the additional signal marked with ▼ was observed only for the positive (intermediate) side in the difference spectra and was tentatively assigned to the signal of the lipid methyl groups, suggesting a structural change in the lipid upon formation of the M-intermediate. The signals marked with ▼ appeared at different positions for the two light conditions (23.9 ppm for 520 nm, 22.0 ppm for 595 nm). We have not ruled out the possibility that the different M-intermediates accumulated under the two light conditions. Indeed, the transient grating method has been used to identify three substrates of M (M1, M2, and M3), which have different partial molecular volumes.[15] In the *pp*R/*p*HtrII system, at least three M-intermediates (M1, M2, and M3) were observed.[10] The origin of the additional signals should be identified in future studies using isotope-labeled lipids and signal temperature dependency. Thus, these results indicate that a M-intermediate at 19.8 ppm was trapped stationary under the photo-illumination conditions, because the half-life of the M-intermediate (750 ms) was relatively long enough to provide a high population in the photocycle as compared with the K- and L-intermediates (270 μs).[5] Owing to their short half-lives, the K- and L-intermediates could not be trapped in the photocycle to the M-intermediate as shown in Figure 1 A,B (see also the Supporting Information, Figure S1).

As a M-intermediate was trapped stationary under illumination with 595 nm LED light, the sample was subsequently irradiated with 365 nm LED light at −40 °C to examine the transition from the M- to the P-intermediate. As shown in Figure 2 A, spectrum c, the signal owing to the M-

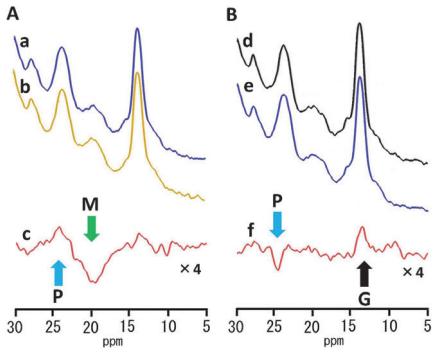

Figure 2. Transformation processes from the M- to P-intermediate and the P-intermediate to the G-state. The ^{13}C NMR signals of [20-^{13}C]retinal-*Sr*SRI-PG system under illumination with 595 nm LED light (yellow; A, spectrum b) allowed accumulation of the M-intermediate. Subsequent illumination with 365 nm LED light allowed accumulation of the P-intermediate (blue; A, spectrum a). The difference spectra of the P- and M-intermediates (a minus b; A, spectrum c) and the relaxation process from the P-intermediate (blue; B, spectrum e) to the G-state (black; B, spectrum d) under subsequent dark conditions and the difference spectrum of the G-state and P-intermediate (d minus e; B, spectrum f).

intermediate at 19.8 ppm decreased and the signal at 24.8 ppm owing to the P-intermediate increased simultaneously.

This result indicates that the M-intermediate is transformed into the P-intermediate by absorbing a second photon of 365 nm LED light (blue). Thus, conversion from the G-state to the P-intermediates via the M-intermediate is a double photon process. The P-intermediate was trapped stationary, as its NMR signals were accumulating for 22 h. This result indicates that the half-life of the P-intermediate (80 ms)[5] should be long enough to provide a large population at −40 °C under illumination with 365 nm LED light. As the ^{13}C chemical shift value of [20-^{13}C]retinal in the P-intermediate was 24.8 ppm, the configuration of retinal should be 13-*cis*, similar to the chemical shift value of *pp*R[10] and BR[11–13] (Supporting Information, Table S1). However, the 13-*cis*, 15-*anti* or 13-*cis*, 15-*syn* configuration was not completely determined in this experiment. This result is contrast to the report that the configuration of the P-intermediate has been tentatively assigned to be *all-trans* in SRI.[6] The current data clearly show the formation of a repellent phototaxis intermediate (P-intermediate) without isomerization of the 13-*cis* (C13 = C14 bond) from the M-intermediate in the retinal chromophore. Because the absorption maximum of the P-intermediate is located at higher wavelength (ca. 525 nm),[8] it is likely that the P-intermediate has a protonated retinal Schiff base. Additionally, as the chemical shift value of the P-intermediate is significantly different from that of the M-intermediate, a rather large conformational change was observed, which may cause large changes in protein interactions, acting as a switch between positive and negative phototaxis functions.[16]

After the P-intermediate was generated and trapped stationary under irradiation with 365 nm LED light of the M-intermediate, light illumination was stopped and the accumulation of the P-intermediate under dark conditions was observed (Figure 2B). The signal intensity of the P-intermediate at 24.8 ppm decreased and that of the G-state at 13.8 ppm increased, indicating that the P-intermediate relaxed to the G-state. It was therefore justified that the P-intermediate returned to the G-state under dark condition by a thermal process.

Although the P-intermediate relaxed to the G-state within a half day under dark conditions at −40 °C, the P-intermediate was trapped stationary as long as it was illuminated with 365 nm LED light (Figure 2A). This indicates that the P-intermediate and the G-state should be equilibrated apparently under the light illumination with 365 nm LED light.

Thus, to examine the pathway from the G-state to the P-intermediate, the G-state was illuminated with 365 nm light (Figure 3A). The result indicated that the G-state decreased

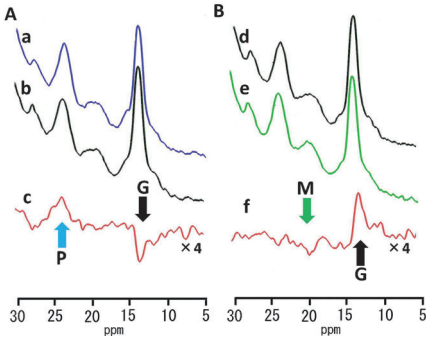

Figure 3. Transformation processes from the G-state to the P-intermediate and from the M-intermediate to the G-state. The ^{13}C NMR signals of [20-^{13}C]retinal-*Sr*SRI-PG system in the dark (black; A, spectrum b) and subsequent illumination with 365 nm LED light led to the accumulation of the P-intermediate (blue; A, spectrum a), and the difference spectrum between the P-intermediate and the G-state (a minus b; A, spectrum c). The relaxation process from the M-intermediate (green; B, spectrum e) to the G-state (black; B, spectrum d) under subsequent dark conditions and the difference spectrum between the G-state and the M-intermediate (d minus e; B, spectrum f).

and that the P-intermediate increased simultaneously (Figure 3A, spectrum c). This result clearly revealed that the P-intermediate was apparently generated directly from the G-state by irradiation with 365 nm LED light. This is discussed further in a later section. Briefly, this can be explained by considering two pathways from the G-state to the P-intermediate (thin blue arrows in Figure 4).

On the other hand, the M-intermediate transformed to the G-state thermally under dark conditions as (Figure 3B). In the difference spectrum (Figure 3B, spectrum f), the intensity of the negative peak is smaller than in the G-state. Although the reason is unclear, this decrease might be caused by equilibrium between multiple M-intermediates, which would induce spectral broadening owing to difference in the peak positions. This result confirmed that SRI follows a typical pathway (without going through the P-intermediate) of a single photon photocycle under the green light illumination in contrast to the pathway followed under UV light irradiation.

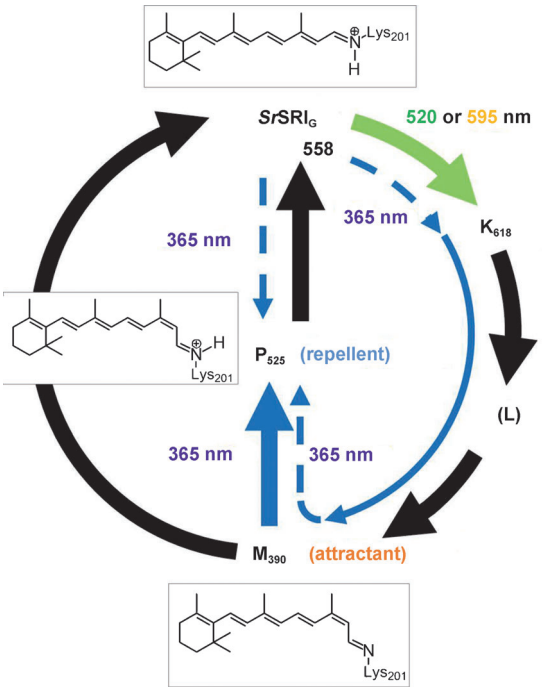

Figure 4. Estimated photoreaction cycle of *Sr*SRI and retinal configurations. The G-state is activated to the K-intermediate under illumination with green and/or orange light (green arrow) followed by transformation to the M-intermediate (attractant) and finally back to the G-state (black arrows; G→(hν)→K→(L)→M→G). Photoactivation of two pathways from the G-state to the P-intermediate (repellent) under illumination with blue light was revealed as single-(thin dotted blue arrow; G→(hν)→P) and double-photon processes (thin dotted and solid blue arrows; G→(hν)→K→(L)→M→(hν)→P). The retinal configuration of the P-intermediate was determined to be 13-*cis* in this study.

These experimental results, revealed the photoreaction cycles and possible configurations of the retinals in the intermediates of *Sr*SRI (Figure 4). In this photoreaction cycle, the M-intermediate was trapped stationary in an attractant state under green light (520 nm) illumination. Subsequently the P-intermediate was trapped stationary by illumination with a second UV light (365 nm) in a repellent state. The configuration of the retinal in the M-intermediate was determined to be 13-*cis*, 15-*anti* with deprotonation of SB (absorption maximum at 390 nm)[8] and in the P-intermediate to be 13-*cis* with protonation of the SB (absorption maximum at 525 nm).[8] In Figure 4, we tentatively assigned the configuration of the P-intermediate to be 13-*cis*, 15-*anti*, although 15-*anti* or 15-*syn* was not determined in this experiment. Interestingly, transformation from the M- to the P-intermediate occurred without retinal isomerization of C13=C14 bond from 13-*cis* to *all-trans*. The pathway from the G-state to the P-intermediate was also observed under illumination with UV light of 365 nm. SRI functions in both positive and negative phototaxis, in contrast to SRII, which regulates only negative phototaxis. SRI acts in positive phototaxis under green light conditions and in negative phototaxis when the light has UV component. This allows the bacteria to escape from a harmful UV light.

To investigate whether or not the G-state absorbs at the wavelength of UV light, we measured the reaction of *Sr*SRI with hydroxylamine. The difference absorption spectra of *Sr*SRI at several time points after the addition of hydroxylamine are shown in the Supporting Information, Figure S2(A). The absorbance of *Sr*SRI at 556 nm decreases and there is a concomitant increase in the absorbance of retinal oxime at 361 nm. Interestingly there was a small negative peak at 429 nm with an isosbestic point at around 400 nm. The absorbance change of *Sr*SRI was plotted against time (Supporting Information, Figure S2(B)). The bleaching rate of the peak at 429 nm (0.00043 min^{-1}) was similar to that at 556 nm (0.00057 min^{-1}), indicating that the peak at 429 nm is a component of the G-state absorbance peak at 558 nm. Thus, it is probable that a small absorbance peak would be found at 365 nm if the absorbance spectrum of the G-state was extrapolated. Consequently, the absorption at 429 nm can be explained by the G→P and/or G→K transitions (Figure 4). Specifically, the G-state can be transformed into the P-intermediate through the K-, L-, and M-intermediates as a double photon process. It is also possible that the G-state can be directly transformed to the P-intermediate as a single photon process. These two pathways can explain why the P-intermediate can be trapped stationary under blue light illumination.

In the scope of this study, we focused on the configurations of the retinal chromophore in *Sr*SRI during the photocycle by observing the chemical shift values of [20-^{13}C]retinal-*Sr*SRI upon illumination with lights of several frequencies. Specifically, we wished to clarify the individual photoreaction pathway and deprotonation state of the M-intermediate. It is also essential to investigate the response of the protein, including the retinal Schiff base. In particular, [ε-^{15}N]Lys is a good probe for distinguishing the protonated from the deprotonated Schiff base.[18] We will monitor the structural changes of *Sr*SRI using the isotope-labeled proteins (for example, ^{15}N-Lys, ^{13}C-Ala, and ^{13}C-Val) in the future.

In conclusion, the photocycle of *Sr*SRI was examined using in situ photo-irradiation solid-state NMR spectroscopy. The M-intermediate was trapped stationary by illumination with 520 or 595 nm LED light at −40 °C and is responsible for the positive phototaxis. Subsequently, the M-intermediate was transformed into the P-intermediate. The P-intermediate was trapped stationary by irradiation with 365 nm LED light and is responsible for the negative phototaxis. The configuration of retinal in the P-intermediate was determined to be in the 13-*cis* form, indicating that the formation of the P-intermediate with protonated SB from the M-intermediate with deprotonated SB occurred without retinal isomerization of 13-*cis* retinal (the C13=C14 bond). It is likely that proton transfer occurs upon formation of the P-intermediate. The G-state was also transformed to the P-intermediate by illumination with 365 nm light. These results clearly indicate that the population of the M-intermediate increases under photo-illumination with green light and functions as an attractant, and the population of the P-intermediate increases under photo-illumination with blue light and functions as a repellent. Consequently, it appears that bacteria can increase the population of the P-intermediate either by a direct single

photon process from the G-state to the P-intermediate, or by a double photon process from the G-state to the P-intermediate via the M-intermediate. In both cases, the bacteria can escape from a harmful ultraviolet light using this color-discriminating process.

Experimental Section

[20-^{13}C]retinal was synthesized using the reported methods.[18] The preparation of crude membranes and the purification of [20-^{13}C]retinal-*Sr*SRI were performed using essentially the same method as previously described.[14]

Photo-irradiation NMR experiments have been reported by several groups.[17,19–21] In the present system, light was illuminated from the inside of the sample rotor through a glass rod inserted into the rotor.[10,22] This illumination system allows us to illuminate the sample with multiple LED lights (520, 595, and 365 nm, Prizmatix, Israel) with extremely high efficiency (Supporting Information, Figure S3).

For the in situ photo-irradiation solid-state NMR measurements, a CP pulse sequence was used with a 1 ms contact time followed by the acquisition using 50 kHz amplitude of TPPM proton decoupling pulses.[23] The temperature was set to −40 °C using a gas flow system for all the dark and LED photo-illumination experiments (see also the Experimental Section in the Supporting Information).

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