Photoreaction pathways and photointermediates of retinal-binding photoreceptor proteins as revealed by in situ photoirradiation solid-state NMR spectroscopy

Akira Naito¹ · Yoshiteru Makino¹ · Arisu Shigeta¹ · Izuru Kawamura¹

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Abstract

Photoirradiation solid-state NMR spectroscopy is a powerful means to study photoreceptor retinal-binding proteins by the detection of short-lived photointermediates to elucidate the photoreaction cycle and photoactivated structural changes. An in situ photoirradiation solid-state NMR apparatus has been developed for the irradiation of samples with extremely high efficiency to enable observation of photointermediates which are stationary trapped states. Such observation enables elucidation of the photoreaction processes of photoreceptor membrane proteins. Therefore, in situ photoirradiation is particularly useful study the photocycle of retinal-binding proteins such as sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII) because functional photointermediates have relatively longer half-lives than other photointermediates. As a result, several photointermediates have been trapped as stationary state and their detailed structures and photoreaction cycles have been revealed using photoirradiation solid-state NMR spectroscopy at low temperature. Photoreaction intermediates of bacteriorhodopsin, which functions to provide light-driven proton pump activity, were difficult to trap because the half-lives of the photointermediates were shorter than those of sensory rhodopsin. Therefore, these photointermediates are trapped in a freeze-trapped state at a very low temperature and the NMR signals were observed using a combination of photoirradiation and dynamic nuclear polarization (DNP) experiments.

Keywords Sensory rhodopsin · Bacteriorhodopsin · Photoreaction cycle · Photointermediate · Photoirradiation solid-state NMR

Introduction

Nuclear magnetic resonance (NMR) spectroscopy provides important information on molecular structure and dynamics, particularly in biological systems. Photoirradiation solid-state NMR spectroscopy is particularly useful for the study of photoreceptor retinal-binding membrane proteins by the detection of short-lived photointermediates, which allows elucidation of the photoreaction cycles and photoactivated structural changes. However, such experiments cannot be performed easily, because photoirradiation systems for solid-state NMR are quite difficult to incorporate due to the complex nature of magic angle spinning (MAS) probe assemblies. Most of the early experiments were performed with photoirradiation from

outside the probe and with freezing of the sample, followed by transfer to the NMR probe for measurement of the signals derived from the trapped photoactivated intermediates (freeze trapping).

Microbial rhodopsin of seven transmembrane helical structures with all-*trans* configuration of retinal chromophore in the dark-adapted state as shown in Fig. 1. In the early years of such research, the light-adapted state and M- and N-intermediates of bacteriorhodopsin (bR) were trapped by photoirradiation with light outside the NMR magnet and characterized the structures using solid-state NMR spectroscopy (Smith et al. 1989; McDermott et al. 1991; Farrar et al. 1993; Lakshimi et al. 1994). In these experiments, photointermediates of bR were trapped at low temperature and the samples were transformed to the NMR probe to observe the NMR signals of photointermediates. The meta-rhodopsin-I photointermediate of rhodopsin was characterized using the photoirradiation method at very low temperature in combination with double-quantum solid-state NMR (Feng et al. 2000). In this experiment, light was irradiated to the inside of the NMR sample tube, in which glass beads were

✉ Akira Naito
naito@ynu.ac.jp

¹ Graduate School of Engineering, Yokohama National University, Yokohama 240-8501, Japan

Fig. 1 Tertiary structure of bacteriorhodopsin (PDB code: 1C3W) and retinal chromophore with all-*trans* configuration in the dark-adapted state

incorporated to disperse the light inside the sample tube. The meta-rhodopsin II photointermediate of the visual receptor rhodopsin was also trapped and characterized using photoirradiation solid-state NMR (Crocker et al. 2006; Ahuja et al. 2009).

An in situ photoirradiation solid-state NMR spectrometer has also been described, in which light passes through an optical fiber in the probe and the sample is irradiated from outside the rotor tube (Hu et al. 1998; Petkova et al. 2002). This system was used to characterize an early M-intermediate, M_0 , in the bR photocycle. The L-intermediate of bR was trapped by irradiation of the light-adapted state (LA) with light at > 610 nm in the temperature range of -95 to -130 °C for at least 2 h (Hu et al. 1997). In combination with the dynamic nuclear polarization (DNP) method, the heterogeneity of dark-adapted bR and distortion in the K-intermediates were revealed, and four discrete L-intermediates were detected (Mak-Jurkauskas et al. 2008; Bajaj et al. 2009). A number of photointermediates of channel rhodopsin-2 were recently generated and insight into the photoactive sites and photoreaction pathways was obtained using DNP-enhanced photoirradiation solid-state NMR (Becker-Baldus et al. 2015).

Another in situ photoirradiation solid-state NMR apparatus was developed, in which the sample is irradiated from inside the rotor tube through a glass rod inserted into the rotor (Kawamura et al. 2007; Tomonaga et al. 2011; Yomoda et al. 2014; Makino et al. 2018; Naito and Kawamura 2014; Naito et al. 2015, 2018a, b; Oshima et al. 2015). This allows irradiation of the sample with extremely high efficiency and enables observation of the photointermediates as stationary trapped states and photoreaction processes of photoreceptor membrane proteins. In situ photoirradiation is particularly useful for study of the photocycles of retinal proteins such as sensory rhodopsin I (SRI) (Yomoda et al. 2014) and sensory

rhodopsin II (SRII), which is also identified as *pharaonis* phoborhodopsin (*ppR*) (Tomonaga et al. 2011; Makino et al. 2018).

This article reviews the recent progress of in situ photoirradiation solid-state NMR to reveal the photoreaction processes and structures of photointermediates in photoreceptor retinal-binding membrane proteins with functions of signal transduction and light-driven proton pumping.

Design of in situ photoirradiation solid-state NMR experiments

An in situ photoirradiation system was developed for solid-state NMR measurement under the MAS condition (Tomonaga et al. 2011). In situ continuous photoirradiation with a light-emitting diode (LED) light source was conducted using an optical fiber passed from outside the magnet through a tightly sealed cap made of a glass rod glued to a zirconia rotor (Fig. 2). Photoirradiation was performed from the top of the spinner without touching the optical fiber to the cap of the spinner. The tip of the glass rod was grained so as to provide irradiation perpendicular to the rotor wall, to which the fluid membrane proteins were attached on the sidewall as a thin film. Therefore, light could be directed perpendicularly from inside the spinner to the inner wall of the rotor. Thus, the light was directed from outside the rotor and provided from inside the rotor, and thus the irradiation efficiency was significantly improved. Using a solid-state NMR spectrometer equipped with photoirradiation system, it is possible to continuously irradiate a sample with three different LED lights such as 150 mW of green (520 nm), orange (590 nm), and UV (365 nm) LED lights.

Fig. 2 Schematic illustration of the in situ photoirradiation solid-state NMR spectrometer. An optical fiber is guided from the outside of the magnet and is connected to an LED light source. The optical fiber is guided from the bottom to the top of the probe head and accurately aligned along the top of the MAS rotor. The top part of the zirconia rotor is capped by a glass rod in which the tip part is ground to provide irradiation perpendicular to the spinner axis, so that strong light is irradiated to the sample from inside the sample tube (Yomoda et al. 2014)

For in situ photoirradiation solid-state NMR measurements, photoreceptor membrane protein samples were placed into a zirconia rotor and sealed with a transparent glass rod as a cap (Fig. 2). A cross-polarization magic angle spinning (CP-MAS) pulse sequence was used with a contact time of 1 ms, followed by acquisition with 50-kHz two-pulse phase modulated (TPPM) proton decoupling pulses (Bennett et al. 1995). It was noted that the ^{13}C NMR signals of [20- ^{13}C]retinal were only observed with the CP pulse sequence because the retinal is located deep inside the core part of the membrane and is immobile. The MAS frequency was set to 4 kHz, and the temperature was set in the range from 0 to -60°C using a gas flow temperature control system.

DNP enhanced in situ photoirradiation solid-state NMR spectroscopy

A DNP spectrometer operated at a field of 9 T (250 GHz for e- and 380 MHz for ^1H frequencies) was used in combination with photoirradiation solid-state NMR (Mak-Jurkauskas et al. 2008; Bajaj et al. 2009; Bajaj et al. 2007). The triple-resonance custom-designed probe used in these experiments was equipped with a wave guide and a multimode optical fiber to supply microwaves and laser light, respectively, to the sample. Photointermediates were generated at an appropriate temperature and the spectra were encoded at 90 K after trapping of the intermediates (freeze trapping).

Microwave irradiation was applied continuously to bR. After ^1H - ^{15}N cross-polarization, the Schiff base (SB) resonance was selected by a soft, band-selective ^{15}N pulse optimized for solid-state NMR (Bajaj et al. 2009). Signals corresponding to the SB were along the z -axis, and all other signals

were allowed to dephase. After rotation to the transverse plane, the ^{15}N magnetization from the SB resonance evolved under the ^{15}N chemical shift during t_1 and was then transformed selectively to retinal-C15 or K216-C ϵ by specially induced filtering in combination with cross-polarization. The ^{15}N and ^{13}C fields were selected to provide spectrally selective, chemical shift-dependent transfer to either directly bonded carbon, and a ramp of 5–6% in the ^{13}C radio frequency field resulted in quasi-adiabatic transfer with excellent efficiency. After the t_2 evolution period under the ^{13}C chemical shift interaction, further correlation was established by homonuclear mixing using proton-driven spin diffusion with a rotary resonance recoupling field or radio frequency-driven recoupling.

The two ^{13}C -labeled spins can be used for double quantum filtering of the spin pair against the natural abundance background, while simultaneously offering the possibility to study the length and dihedral angle of the C14-C15 bond in retinal-binding membrane proteins such as bR (Feng et al. 2000; Concistrè et al. 2008; Becker-Baldus et al. 2015).

Stationary trapped photointermediate by in situ photoirradiation solid-state NMR spectroscopy

When retinal-binding membrane proteins, such as SRII, are irradiated with LED light, retinal absorbs the light energy and is excited from the G-state to the K-intermediate, subsequently relaxes to L-, M-, and O-intermediates and finally returns to the G-state as follows: $G \rightarrow K \rightarrow L \rightarrow M \rightarrow O \rightarrow G$, which is referred to as a photocycle (Fig. 3A). It is important to note that the M-intermediate exhibits a significantly longer half-life than the other states in the case of sensory rhodopsin;

Fig. 3 Photoreaction cycle and half-lives of photointermediates of ppR (SRII). The half-life of M₃₉₀-intermediate is 1.7 s. **A** Photoreaction cycle of SRII. **B** (a) ¹³C CP-MAS NMR spectrum for dark state (dark). (b) ¹³C CP-MAS NMR spectrum under irradiation with green light (green). (c)

Difference spectrum (dark–green). **C** (a) ¹³C CP-MAS NMR spectrum under irradiation with green light. (b) ¹³C CP-MAS NMR spectrum after turning off the green light (dark 2). (c) Difference spectrum (green–dark 2)

therefore, the M-intermediates can be stationary trapped under continuous light irradiation because the light-activated production rate of the M-intermediate is possibly faster than the relaxation rate. Thus, in a system with a photocycle, photoactivation process can be observed by accumulating ¹³C CP-MAS NMR spectra firstly under dark condition (Fig. 3B (a)) and subsequently under irradiation with LED green light (Fig. 3B (b)) using the same experimental parameters such as the temperature. The difference spectrum between the dark and green light conditions provides a background-free spectrum in which reactant species exhibit negative peaks and product species exhibit positive peaks (Fig. 3B (c)). The relaxation process from the green light state to the dark state can be observed in the difference spectrum of (green–dark 2) as shown in Fig. 3C (c). Thus, the photoreaction pathways can be clearly characterized. It is also possible to switch the photoreaction pathways by changing the wavelength of the LED light. In case of the SRII (ppR) system, the M-intermediate may switch pathways from M (390 nm) → O (560 nm) to M (390 nm) → SRII_G under irradiation with UV light (365 nm).

Photoreaction cycle of *pharaonis* phoborhodopsin (ppR or also called SRII) revealed by in situ photoirradiation solid-state NMR spectroscopy

SR-II, which is known as ppR is a retinal-binding membrane protein isolated from the halophilic and alkaliphilic archaeal *Natronomonas pharaonis*, which consists of seven-transmembrane α-helices with a vitamin A aldehyde retinal

as a chromophore (Govorunova et al. 2017). All-*trans* retinal is predominantly incorporated into the apoprotein of ppR. ppR functions as a photoreceptor protein by forming a 2:2 complex with the cognate two-helix transducer protein *pharaonis* halobacterial transducer II (pHtrII) to transmit the photosignal into the cytoplasm (Hoff et al. 1997; Spudich and Lucke 2002; Suzuki et al. 2010; Inoue et al. 2014). The ppR/pHtrII complex is involved in negative phototaxis through a cyclic photoreaction pathway called the photocycle (Fig. 3A). In the initial state, ppR has an absorbance maximum at 498 nm in the ground (G) state under dark conditions. Light absorption transforms ppR from the ground state (ppR_G) to a K-intermediate (540 nm) with an absorbance maximum of 540 nm. This transformation is initiated by *trans* to *cis* photoisomerization of the retinal. The photocycle is followed by several intermediates, such as L (498 nm), M (390 nm), and O (560 nm), undergoing thermal relaxation processes. Finally, the O-intermediate thermally returns to the ppR_G state (Kamo et al. 2001).

The K-intermediate has a half-life of ca. 1 μs, and its retinal is in the 13-*cis*, 15-*anti* configuration. Relaxation of the K-intermediate provides the L-intermediate, which has a half-life of ca. 30 μs and a 13-*cis*, 15-*anti* retinal configuration (Fig. 3A). A proton is subsequently removed from the SB of the L-intermediate, which results in transformation to the M-intermediate, which has a long half-life of ca. 1.7 s. The M-intermediate has a 13-*cis*, 15-*anti* retinal configuration with a deprotonated SB. Upon reprotonation, the M-intermediate transforms into the O-intermediate with a protonated SB, which has a half-life of 770 ms and a 13-*trans*, 13-*syn* retinal configuration (Fig. 3A) (Suzuki et al. 2010; Inoue et al. 2014; Kamo et al. 2001; Imamoto et al. 1992). The M- and O-

intermediates have longer half-lives than the K- and L-intermediates, and thus they are known as late-active intermediates.

Important signal transduction processes such as changes in protein structure are induced at the late step in the photocycle. These changes likely include the formation of two specific hydrogen bonds, one between Tyr199_{ppR} and Asn74_{pHtrII} and one between Thr189_{ppR} and Glu43_{pHtrII}/Ser62_{pHtrII}, as observed in the crystal structure of the ppR/pHtrII complex in the dark state (Gordeliy et al. 2002) and the K- and M-intermediates (Moukhametzianov et al. 2006; Ishchenko et al. 2017). Thr204 is another important residue in ppR that plays a role in color tuning and in the photocycle kinetics of ppR (Shimono et al. 2003). Further observations have shown that Thr204 is indispensable for the negative phototaxis function of the complex (Sudo et al. 2006). There is steric hindrance in the K-intermediate between the C14-H of retinal and Thr204 (Sudo et al. 2005). At the same time, a specific hydrogen bond alteration occurs between Thr204 and Thr174 in a pHtrII-dependent manner (Sudo et al. 2006). Helix movement in ppR and outward tilting of helix F during the photocycle have been suggested by various groups (Spudich 1998; Wegener et al. 2000; Yoshida et al. 2004) and are believed to be essential steps for the activation of pHtrII. However, no helix tilting was observed in the crystal structure of the M-intermediate of the ppR/pHtrII complex (Moukhametzianov et al. 2006). After tilting of the F-helix in ppR, TM2 in pHtrII rotates and transfers the signal to the phosphorylation cascade to initiate rotation of the bacterial flagellar motor, which results in negative phototaxis.

The retinal chromophore in ppR forms a covalent bond with a perfectly conserved Lys residue (Lys205) bonded to the SB. Animal rhodopsin (type II rhodopsin) photobleaches, whereas microbial rhodopsin (type I rhodopsin) retains the retinal through the photocycle. Therefore, continuous irradiation of ppR with green light results in repeated photocycles without photobleaching, and the photointermediates can be trapped in a stationary state using a type I rhodopsin such as ppR (Feng et al. 2000; Tomonaga et al. 2011; Ernst et al. 2014).

Photoreaction pathways under green light irradiation of the ppR/pHtrII complex

In situ photoirradiation solid-state ¹³C CP-MAS measurements were performed on the [20-¹³C]-labeled retinal-ppR/pHtrII complex at −40 °C (Makino et al. 2018). Photoreaction pathways under irradiation with green light were obtained by first observing the ¹³C CP-MAS NMR spectrum of the ppR/pHtrII complex under the dark condition (dark 1) at −40 °C (Fig. 4A (a)), and then the ¹³C CP-MAS

spectrum under irradiation with 520 nm (Fig. 4A (b)) to observe the intermediates in the stationary state.

The [20-¹³C]retinal signals shown in Fig. 4A heavily overlapped with the lipid signals. The difference spectrum (green–dark 1) was therefore obtained as shown in Fig. 4B (d) to analyze the photoreaction pathways induced by photoirradiation. The ¹³C CP-MAS NMR signal of the G-state at 13.5 ppm (reactant state) decreased, and those of the M-intermediates at 22.1 and 22.9 ppm (*M*₁ and *M*₂), the N'-intermediates at 23.9 ppm, and the O-intermediate at 16.1 ppm (product states) all increased (Fig. 4B (d)). Thus, the ¹³C CP-MAS NMR signals of the [20-¹³C]retinal-ppR/pHtrII complex show the process of photoisomerization from the G-state (13.5 ppm) to a number of photointermediates. The photointermediates in the 13-*cis* form (22.9, 22.1, and 23.8 ppm) were assigned to the *M*₁, *M*₂, and N'-intermediates, respectively, though the signal at 23.8 ppm was previously assigned to the *M*₃ intermediate (Tomonaga et al. 2011). The photointermediate in the 13-*trans* form (16.1 ppm) was assigned to the O-intermediate. In summary, the 13-*trans* form product peak at 16.1 ppm can be assigned to the O-intermediate, and the 13-*cis* form can be assigned to the M-intermediates (22.1, 22.9 ppm) and the N'-intermediate (23.0 ppm) (Fig. 4C and Table 1).

Relaxation process from the green light irradiation state to the short dark state

The green light irradiation state was relaxed to the dark state by the process of thermal relaxation. The ¹³C CP-MAS NMR spectrum of the ppR/pHtrII complex was obtained 1 day after termination of the green light irradiation. The difference spectrum shown in Fig. 4B (e) was obtained by subtracting the initial dark state (dark 1) from the second short dark state (dark 2). The peak at 23.9 ppm remained unchanged, whereas the peaks at 22.1 and 22.9 ppm decreased in intensity. The difference spectrum shown in Fig. 4B (f) was obtained by subtracting the green light irradiation state (green) from the second short dark state (dark 2). The peaks at 22.1, 22.9, and 16.1 ppm decreased, and the G-state increased in intensity. These results indicate that the peak at 23.9 ppm is not due to the M-intermediates but rather to the N'-intermediate, and therefore the half-lives of the M-intermediates at 22.1 and 22.9 ppm and of the O-intermediate at 16.1 ppm are much shorter than that of the N'-intermediate (N-like intermediate), which relaxed more slowly than the M- and O-intermediates.

Photoreaction pathway from the M-intermediates to the O-intermediate

An N-like intermediate with an absorbance maximum at 500 nm was previously observed in ppR in the 13-*cis* form

Fig. 4 Photoreaction pathways of *ppR/pHtrII* complex. **A** ^{13}C CP-MAS NMR spectra under (a) dark1 condition, (b) green light irradiation, and (c) under dark 2 condition. **B** Difference spectra: (d) (green–dark 1), (e) (dark2–dark 1), and (f) (dark 2–green). **C** Photoreaction cycle of *ppR/pHtrII* complex determined by green light irradiation. **D** Difference

spectra: (a) (green–dark 1), (b) (UV–dark 1), and (c) (UV–green). **E** Difference spectra: (d) (UV–dark 1), (e) (dark 1–dark 2), and (f) (dark2–UV). **F** Photoreaction cycle of *ppR/pHtrII* complex determined by green and UV light irradiation (Makino et al. 2018)

by a transient absorption study (Chizov et al. 1998), and subsequently the N-like intermediate was observed using azide to accelerate the decay of the M-intermediates (Tateishi et al. 2011). After accumulation of the M-intermediate by irradiation with 520-nm light, the sample was irradiated with UV light at 365 nm to eliminate the M-intermediate (maximal absorbance of 390 nm) that allowed to confirm the existence of this N-like intermediate.

After the M-intermediates of the *ppR/pHtrII* complex were trapped at -40°C in a stationary manner by irradiation with light at 520 nm, the green light was then switched to 365 nm UV light (Fig. 4F). During this process, the intensities of the M-intermediates decayed and the intensities of the O-intermediate and the G-state increased (Fig. 4D (c)), whereas the N'-intermediate did not decay over the same period. Furthermore, it is noted that the M-intermediates were transformed to the O-intermediate after irradiation with 365-nm UV light (Fig. 4D (c)). The M-intermediates are reportedly

transformed back to the G-state by irradiation with UV light (365 nm) (Roy et al. 2006), because the M-intermediates have a maximal absorption of 390 nm. However, this experiment revealed that the M-intermediates are transformed to the O-intermediate upon irradiation with 365-nm UV light (Fig. 4D (c)) (Makino et al. 2018). Spectroscopic evidence for the formation of an N'-intermediate was recently obtained in a transient absorption study (Tateishi et al. 2011), which reported that the decay of the M-intermediates does not directly produce the N'-intermediate, but rather produces an O-intermediate that is in equilibrium with the N'-intermediate. In contrast to the case of bR, the N'-intermediate was transformed from the O-intermediate, and this intermediate is thus named an N'-intermediate rather than an N-intermediate. Based on previously reported findings and the result of the in situ photoirradiation solid-state NMR experiments (Makino et al. 2018), the photoreaction cycle of the *ppR/pHtrII* complex can be summarized as shown

Table 1 ^{13}C chemical shifts of individual states of [20- ^{13}C , 14- ^{13}C]-retinal-sensory rhodopsin proteins (ppm)

Protein	State	20- ^{13}C	14- ^{13}C	Configuration
<i>ppR</i> (0 °C) ^a	G-state	13.3		13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	M-intermediate	22.3		13- <i>cis</i>
<i>ppR</i> (−20 °C) ^a	G-state	13.5		13- <i>trans</i>
	M-intermediate	24.1, 22.5, 21.7		13- <i>cis</i>
<i>ppR/pHtrII</i> (−40 °C) ^b	G-state	13.5		13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	M-intermediate	22.1, 22.9		13- <i>cis</i>
	O-intermediate	16.1		13- <i>trans</i>
	N'-intermediate	23.9		13- <i>trans</i>
<i>ppR</i> (−40 °C) ^b	G-state	13.5	121.7	13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	M-intermediate	22.3	126.8	13- <i>cis</i> , 15- <i>anti</i>
<i>ppR</i> (−60 °C) ^b	G-state	13.6	121.6	13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	M-intermediate	22.6	127.1	13- <i>cis</i> , 15- <i>anti</i>
	O-intermediate	16.4	115.4	13- <i>trans</i> , 15- <i>syn</i>
	N'-intermediate	23.9	115.4	13- <i>cis</i>
<i>SrSRI</i> (−40 °C) ^c	G-state	13.8		13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	M-state	19.8		13- <i>cis</i>
	P-state	24.8		13- <i>cis</i>

^a Tomonaga et al. 2011^b Makino et al. 2018^c Yomoda et al. 2014

in Fig. 4F. The M-intermediates are converted to the O-intermediate under irradiation with 365-nm UV light through a double-photon process, and the O-intermediate is then converted to the N'-intermediate until an equilibrium state between the O- and N'-intermediates is achieved, in which the intensity of the N'-intermediate is higher than that of the O-intermediate (Fig. 4E (d)).

Retinal configuration of the late photointermediates of the *ppR* and *ppR/pHtrII* complex

^{13}C chemical shifts of the late photointermediates, M-, O-, and N'-intermediate were clearly determined by observation of the ^{13}C NMR signals by photoirradiation solid-state NMR, as summarized in Fig. 4F and Table 1. ^{13}C chemical shifts of the 13-C position reflects the C13 = C14 configuration, i.e., the chemical shifts of 12–16 ppm indicate 13-*trans* and those of 18–25 ppm indicate 13-*cis* configurations. On the other hand, ^{13}C chemical shifts of the 14-C position reflect the C15=N ζ configuration, i.e., 113–116 ppm indicates the 15-*syn* configuration and 118–128 ppm indicates the 15-*anti* configuration (Table 1). Using these relationships, the configurations of the M-, O-, and N'-intermediates were determined to be (13-*cis*, 15-*anti*), (13-*trans*, 15-*syn*), and (13-*cis*),

respectively (Fig. 4F). In the case of the N'-intermediate, although the 14-C chemical shifts was 115.4 ppm, the C15 = N ζ configuration was not determined to be 15-*syn* by examination with the density functional theory (DFT) calculations (Makino et al. 2018).

Photoreaction cycle of SRI revealed by in situ photoirradiation solid-state NMR spectroscopy

SRI is a membrane-embedded microbial rhodopsin and that functions as a color-discriminating photoreceptor protein in the halobacterial phototaxis (Spudich and Bogomolni 1984). SRI has seven transmembrane α -helices and has all-*trans* retinal chromophore in the dark state (Hoff et al. 1997; Suzuki et al. 2010). SRI forms a 2:2 complex with its cognate transducer protein, halobacterial transducer protein for SRI, and HtrI (Chen and Spudich 2002). The essential photocycle of SRI from *Halobacterium salinarum* (*HsSRI*) proceeds as follows: SRI_G(587 nm) $\rightarrow$ (h ν) $\rightarrow$ SRI_K(620 nm) $\rightarrow$ SRI_L(540 nm) $\rightarrow$ SRI_M(373 nm) $\rightarrow$ SRI_G(587 nm). The SRI_L(520 nm) intermediate is produced from the M-intermediate by a second photon absorption of UV light

(365 nm) as a back reaction to SRI_G , i.e., $\text{SRI}_M(373 \text{ nm}) \rightarrow (\text{h}\nu) \rightarrow \text{SRI}_P(520 \text{ nm}) \rightarrow \text{SRI}_G(587 \text{ nm})$ (Swartz et al. 2000). During the photocycle, the M- and P-intermediates are considered to be essential for positive and negative phototaxis, respectively.

A 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 light) generates a repellent signal (P-intermediate), which induces flagellar reversal, as compared to the non-signaling state (G-state) (Spudich and Bogomolni 1984). Thus, the structural changes of both the protein moiety and the retinal chromophore upon formation of the active M- and P-intermediates continue to provide important information.

In situ photoirradiation solid-state NMR experiments revealed the photoreaction cycle and possible configurations of the retinal in the intermediates of *SrSRI* from *Salinibacter ruber* as shown in Fig. 5E. In this photoreaction cycle, the M-intermediate was trapped stationary in an attractant state under green light (520 nm) irradiation (Fig. 5A). On the other hand, the P-intermediate was trapped stationary by irradiation with UV light (365 nm) in a repellent state (Fig. 5B). Further, the M-intermediate transformed into the P-intermediate by switching from green to UV light (Fig. 5C). The ^{13}C chemical shifts of the 20- ^{13}C position in the M-intermediate appeared at 19.8 ppm. Therefore, the configuration of the retinal in the

M-intermediate was determined to be 13-*cis*, 15-*anti* with deprotonation of the SB (absorption maximum at 390 nm) (Kitajima-Ihara et al. 2008). The ^{13}C chemical shifts of the 20- ^{13}C position of the P-intermediate appeared at 24.8 ppm. Therefore, the configuration of the P-intermediate was determined to be 13-*cis* with protonation of the SB (absorption maximum at 525 nm) (Kitajima-Ihara et al. 2008). As shown in Table 1 and Fig. 5E, the configuration of the P-intermediate was assigned to 13-*cis*, 15-*anti*, although 15-*anti* or 15-*syn* were not determined in this experiment. It should be noted that transformation from the M- to the P-intermediate occurred without retinal isomerization of the C13 = C14 bond from 13-*cis* to all-*trans*. SRI functions with both positive and negative phototaxis, in contrast to SRII, which regulates only negative phototaxis. SRI acts with positive phototaxis under green light conditions and with negative phototaxis when the light has a UV component. These results clearly indicate that the population of the M-intermediate increases under photoirradiation with green light and functions as an attractant, while the population of the P-intermediate increases under photoirradiation with UV 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 harmful UV light using this color discriminating process.

Fig. 5 Photoreaction pathways of SRI from *Salinibacter ruber* (*SrSRI*). **A** ^{13}C CP-MAS NMR spectra of $[20-^{13}\text{C}]\text{ret-SRI}$ taken in the dark (top), green light irradiation (top), and green–dark (bottom). **B** ^{13}C CP-MAS NMR spectra of $[20-^{13}\text{C}]\text{ret-SRI}$ taken in the dark (top), UV light irradiation (top), and UV–dark (bottom). **C** ^{13}C CP-MAS NMR spectra of $[20-^{13}\text{C}]\text{ret-SRI}$ taken in the green light (top), UV light irradiation (top), and UV–green (bottom). **D** ^{13}C CP-MAS NMR spectra of $[20-^{13}\text{C}]\text{ret-SRI}$ taken in the UV light (top), dark 2 (top), and dark 2–UV (bottom). **E** Photoreaction cycle of SRI (Yomoda et al. 2014)

Photoreaction cycle of bR revealed by photoirradiation solid-state NMR spectroscopy

bR is a retinal-binding transmembrane H^+ pump found in the purple membrane (PM) of *Halobacterium salinarum*, where it generates a proton gradient across the cytoplasmic membrane for ATP synthesis (Lanyi 1997). The 26-kDa bR protein contains seven transmembrane α -helices and a retinal group covalently bonded to the ϵ -amino group of Lys216 in helix G. In the extracellular region, an extensive three-dimensional hydrogen-bonded network of protein residues and seven water molecules connects the buried retinal SB and the proton acceptor Asp85 to the membrane surface (Luecke et al. 1999). The energy from the light absorbed by the protein causes isomerization of the retinal configuration from all-trans to 13-*cis*, 15-*anti* (Fig. 6C), which in turn induces conformational changes in the protein, including a change in the tilt angle of the E and F helices. The structural change suggests that protonation of Asp85 initiates a cascade of atomic displacements in the extracellular region, which then causes the release of a proton to the surface (Luecke et al. 2000). Changes in the packing of the helices alter the exposure of a series of charged residues within the protein, including Asp85, Asp96, Glu194, and Glu204, which causes stepwise changes in the pKa values of the side chain up to 3 pH units and results in a H^+ flux to the extracellular side of the protein (Lanyi 2006). Recently, time-resolved serial femtosecond crystallographic structural study of bR using an X-ray-free electron laser provided some structural events that occur in the nanoseconds and milliseconds after photoactivation (Nango et al. 2016). This time-resolved structural analyses revealed how structural changes in bR achieve up directional membrane transport of H^+ .

The ordered sequence of conformational changes that occur in bR as a result of light absorption is known as the photocycle (Fig. 6C), which is started from a dark-adapted state that consists of bR with all-trans (AT, bR₅₆₈) and 13-*cis*, 15-*syn* (CS; bR₅₅₅) retinal configurations with a molar ratio close to 1. The AT state takes the photocycle (AT photocycle) through five additional major distinct states (i.e., the K-, L-, M-, N-, and O-intermediates) which are characterized by changes in the wavelength of maximum absorbance, and finally the AT photocycle returns to the AT state (Fig. 6C) (Lanyi 2000). In contrast, the photocycle of the CS state (CS photocycle) has not been extensively studied. The CS state was irradiated with a Nd laser (530 nm), and an intermediate with a maximum absorbance of 610 nm was detected. The same intermediate was not detected in the AT photocycle; therefore, it was determined to be an intermediate of the CS state and assigned as batho-13-*cis*-bR (Iwasa et al. 1981), “the 610”-intermediate (Kalisky et al. 1977) or K^D-intermediate

(Roepe et al. 1988). The intermediates are subsequently converted to the AT state through a leaking process from the CS photocycle to generate a light-adapted state.

During the transition from the L- to M-intermediate in the AT photocycle, H^+ is transported from a SB to Asp85 via W402, and H^+ is simultaneously released from a proton release group (PRG), such as Glu194 or Glu204 at the extracellular side. In the transition from the M- to N-intermediate, H^+ is transported from Asp96 to a deprotonated SB to form a protonated SB (SBH). During the transition from the N- to the O-intermediate, H^+ is transported from the cytoplasmic side to Asp96, which causes uptake from the cytoplasmic side to bR (Morgan et al. 2012).

Solid-state NMR spectroscopy represents a powerful method for the detection of intermediates during the photocycle. However, the half-lives of the photointermediates of bR are much shorter than those of SRI and SRII. The L-, M-, and N-intermediates have been detected by measurement of the ^{13}C NMR signals of [^{13}C]retinal (McDermott et al. 1991; Farrar et al. 1993; Lakshmi et al. 1994) and the ^{15}N NMR signals of [ζ - ^{15}N]Lys-bR (Hu et al. 1998, 1997; Mak-Jurkauskas et al. 2008). The 13-*cis* form of the [20- ^{13}C]retinal can be distinguished from the 13-*trans* form, and the 15-*syn* form of [14- ^{13}C]retinal can be distinguished from the 15-*anti* configuration based on the γ -position effects (Smith et al. 1989; Hu et al. 1998; Bajaj et al. 2009). In another study, two protein structures that correspond to the AT and CS retinal configuration were detected by observing rotational echo double resonance (REDOR) filtered ^{13}C NMR signals of [1- ^{13}C]Tyr185-bR (Kawamura et al. 2007).

Photoreaction pathways of bR revealed by DNP-enhanced photoirradiation solid-state NMR spectroscopy

1D DNP solid-state ^{15}N CP-MAS NMR spectra of [ζ - ^{15}N]Lys-bR were recently measured (Fig. 6A) (Mak-Jurkauskas et al. 2008). The spectrum of the dark-adapted sample shows bR₅₅₅ and bR₅₆₈ in a ratio of 60:40. Subsequent irradiation at 275 K converted bR₅₅₅ to bR₅₆₈. Irradiation at 90 K produced two new signals. The major product can be assigned to the K-state observed at 156.5 ppm (Fig. 6A (c)). Thermal relaxation (in the dark) at 150–170 K gives rise to the L-intermediate observed in the functional photocycle. At 150 K, the photocycle proceeds beyond the K-intermediate, and L-intermediates are accumulated. The most downfield signal was assigned to the functional L-state, based on the wavelength dependence of the intensity and thermal relaxation in the M-state. Four L-intermediates were distinguished as L_{165} , L_{174} , L_{181} , and L_{185} , according to the ^{15}N chemical shifts (Fig. 6A; Table 2).

At higher temperature, the photocycle progresses beyond the L-intermediates and the M-intermediates are accumulated. The far-downfield signal of the SB reflects the deprotonated state at this stage of the photocycle. At 210 K, the major

product is the early M-intermediate, M_0 , at 318 ppm. Warming to 260 K yields the late M-intermediate, M_n , at 312 ppm (Fig. 6A (f); Table 2). Thus, 1D DNP solid-state MAS NMR spectra indicated the K-intermediate and a

subsequent increase of temperature generated the four distinct L-intermediate, which were further changed to M_0 - and M_n -intermediates in the photocycle of bR.

DNP-enhanced 2D MAS NMR spectra of bR intermediates acquired from U- ^{13}C ; ^{15}N -labeled samples have been reported (Bajaj et al. 2009). The resolution afforded by chemical shift dispersion in the 2D ^{15}N - ^{13}C correlation MAS NMR spectra of cryogenically trapped photocycle intermediates allow identification of the cross-peaks belonging to unreacted bR568, in addition to several other components (Fig. 6B). There was evidence of multiple intermediates in previous 1D spectra (Hu et al. 1997; Mak-Jurkauskas et al. 2008) and the signal-to-noise ratio in the DNP-enhanced spectra, and the 2D resolution provide additional supporting evidence for their presence. For example, the 2D spectra reveal unexpected heterogeneity in dark-adapted bR, distortion in the K-state, and 4 discrete L-sub-states (Figs. 6A (c) and (d); Table 2). Temperature-dependent studies indicate that only one of these L- sub-states is involved in ion translocation, whereas the other three sub-states are shunts that return to bR₅₆₈. The functional L-state is distinguished from the shunt states by a very strong counter ion interaction and planarization of the chromophore around the isomerized C13 = C14 bond.

Examination of the ^{15}N - ^{13}C 2D correlation spectra indicates single ^{15}N - ^{13}C cross peaks for each carbon atom in the light adapted, K, and M_0 states. In contrast, the spectra of the

dark-adapted and L-states indicate heterogeneity. Dark adaptation is generally considered to involve the equilibrant all-*trans*, 13-*trans*, 15-*anti* bR₅₆₈ and 13-*cis*, and 15-*syn* bR₅₅₅ configurations, and 2 lines with a 40:60 intensity ratio (Mak-Jurkauskas et al. 2008) were observed in 1D ^{15}N spectra and assigned to these species with ^{15}N chemical shifts observed (Fig. 6A (a)) that reflect 2 distinct environments for the SB (Table 2). However, the stronger signals clearly indicate multiple ^{15}N - ^{13}C cross peaks for each species in the ^{13}C dimension (Fig. 6B) (Bajaj et al. 2009). For the less-shielded SB (bR₅₅₅), additional ^{15}N - ^{13}C 15 splitting is particularly evident and indicates well-defined chromophore isomerization with an intensity ratio of ca. 2:1 (Bajaj et al. 2007). The splitting in both the bR₅₅₅ and bR₅₆₈ components of the dark-adapted mixture indicates that each represents at least two slightly different conformations in the neighborhood of the SB linkage. In contrast, these four lines coalesce to a single homogeneous species in the functional bR₅₆₈ obtained after light adaptation.

Overall, the manifold of distinct ^{15}N - ^{13}C cross peaks suggests an ensemble of well-defined L-sub-states rather than a conformationally disordered state. When this 150 K mixture of sub-states (each identified by a subscript with its ^{15}N chemical shift) is warmed to 170 K, the L_{186} signal persists and the signals from the other three L-sub-states decrease, whereas the bR₅₆₈ signals increase. Thus, it is concluded that the L_{166} ,

Table 2 ^{13}C and ^{15}N chemical shift values of individual states of [20- ^{13}C , 14- ^{13}C , 15- ^{13}C]-retinal- and [^{15}N]-Lys-bacteriorhodopsin proteins (ppm)

Protein	State	20- ^{13}C	14- ^{13}C	15- ^{13}C	^{15}N	Configuration
bR	AT (bR568)	13.3 ^a	122.0 ^a	160.0 ^a	165.2 ^e	13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	CS (bR548)	22.0 ^a	110.5 ^a	163.2 ^a	173.5 ^e	13- <i>cis</i> , 15- <i>syn</i>
	K		118.2 ^d	160.2 ^d	156.5 ^e	13- <i>cis</i> , 15- <i>anti</i>
	L165	15.5 ^d		163.0 ^d	165.2 ^e	13- <i>cis</i> , 15- <i>anti</i>
	L174		123.3 ^d	162.9 ^d	174.3 ^e	13- <i>cis</i> , 15- <i>anti</i>
	L181	24.0 ^d	116.2 ^d	162.5 ^d	181.2 ^e	13- <i>cis</i> , 15- <i>anti</i>
	L185	24.5 ^d	116.2 ^d	162.7 ^d	184.9 ^e	13- <i>cis</i> , 15- <i>anti</i>
	M_0	21.5 ^d , 19.6 ^g	124.5 ^d	165.4 ^d	318.4 ^e	13- <i>cis</i> , 15- <i>anti</i>
	M_n	17.8 ^g	124.6 ^c		312.0 ^e	13- <i>cis</i> , 15- <i>anti</i>
	N		115.2 ^b		150.7 ^b	13- <i>cis</i> , 15- <i>syn</i>
Y185F-bR	AT	13.2 ^f	123.1 ^f	159.4 ^f		13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)
	CS	21.7 ^f	110.0 ^f	165.4 ^f		13- <i>cis</i> , 15- <i>syn</i>
	CS*	18.0 ^f	115.3 ^f	162.2 ^f		13- <i>cis</i> , 15- <i>syn</i>
	N	19.2 ^f	125.4 ^f	163.8 ^f		13- <i>cis</i> , 15- <i>anti</i>
	O	13.2 ^f	123.1 ^f	159.4 ^f		13- <i>trans</i> , 15- <i>anti</i> (all- <i>trans</i>)

^a Smith et al. 1989

^b Lakshimi et al. 1994

^c Hu et al. 1998

^d Bajaj et al. 2009

^e Mak-Jurkauskas et al. 2008

^f Oshima et al. 2015

^g Petkova et al. 2002

L_{174} , and L_{181} states contribute to the L-to-bR back reaction. However, L_{186} is the functional L-state (Mak-Jurkauskas et al. 2008).

In the ^{13}C dimension of the ^{15}N - ^{13}C 2D correlation spectra (Fig. 6B; Table 2), chemical shifts for 20- ^{13}C and 14- ^{13}C under photoirradiation. These chemical shifts enabled assignments of retinal configuration for photointermediates. Thus, all L-intermediates exhibited 13-*cis* and 15-*anti* configurations (Table 2).

Photoreaction pathways of Y185F-bR mutant revealed by in situ photoirradiation solid-state NMR spectroscopy

In the Y185F-bR mutant, the CS-AT molar ratio in the dark-adapted state is ca. 2:1 (Duñach et al. 1990). Light adaptation produces the AT state and the O-intermediate (Sonar et al. 1993). Photoexcitation of the O^{Y185F}-intermediate at 170 and 250 K produces an N-intermediate, but no M-intermediate (He et al. 1993). In the process of transition from

the O-intermediate to the AT state, H^+ is transported from Asp85 to a proton release group (PRG) through the Y185F-bR hydrogen bond network, which consists of Asp212 and Tyr185. The breakdown of the hydration bond network prevents H^+ transport; therefore, it is predicted that the half-life of the O-intermediate would be significantly increased (Rath et al. 1993).

The photoreaction pathways of Y185F-bR were examined using in situ photoirradiation solid-state NMR spectroscopy (Oshima et al. 2015). ^{13}C CP-MAS NMR spectra were acquired at -40°C in the dark (D1), under irradiation with 520-nm light (L1), subsequently in the dark (D2), and again under irradiation with 520-nm light (L2) (Fig. 7A). Between D1 and L1, the CS (21.7 ppm for 20- ^{13}C and 110.0 ppm for 14- ^{13}C ; bR₅₄₈) state changed to a CS* (18.0 ppm for 20- ^{13}C and 115.0 ppm for 14- ^{13}C ; 13-*cis*, 15-*syn*)-intermediate, which was highly stable at -40°C and was similar to the batho-13-*cis*-bR intermediate (Iwasa et al. 1981) and K^D-intermediates (Roepe et al. 1988) (Fig. 7A (a); Table 2). The AT (13.2 ppm for 20- ^{13}C and 123.1 ppm for 14- ^{13}C ; bR₅₆₈) state

Fig. 7 Photoreaction cycle of Y185F-bR. **A** ^{13}C CP MAS difference spectra of [20- ^{13}C]Ret-Y185F-bR at -40°C . (a) Subtracting dark 1 state (D1) from light 1 state (L1). (b) Subtracting light 1 state (L1) from dark 2 state (D2). (c) Subtracting dark 2 state (D2) from light 2 state (L2). (d) Subtracting light 2 state (L2) from dark 3 state (D3). (e) Subtracting dark

1 state (D1) from dark 2 state (D2) (Oshima et al. 2015). **B** Photoreaction cycle of Y185F-bR. Solid green arrows indicate the photoreaction pathways under irradiation with 520-nm green light. Solid black arrows indicate thermal relaxation pathways. Broken arrows indicate possible thermal pathway (Oshima et al. 2015)

transformed to an N-intermediate (13.2 ppm for $20\text{-}^{13}\text{C}$ and 125.4 ppm for $14\text{-}^{13}\text{C}$) (Fig. 7A (a)). Under the D2 condition, the N-intermediate transformed into an O-intermediate (13.2 ppm for $20\text{-}^{13}\text{C}$ and 123.1 ppm for $14\text{-}^{13}\text{C}$), which was also highly stable at $-40\text{ }^{\circ}\text{C}$ in the dark (Fig. 7A (b)). Consequently, the O-intermediate transformed into the N-intermediate through the AT state, whereas the CS^* -intermediate did not change under irradiation with 520-nm light (Fig. 7A (c)). The CS^* -intermediate was converted to the AT state after the temperature was increased to $-20\text{ }^{\circ}\text{C}$. Upon subsequent increase of the temperature to $20\text{ }^{\circ}\text{C}$, the AT state was converted to the CS state until equilibrium was reached. In this experiment, the chemical shifts of the $[20\text{-}^{13}\text{C}, 14\text{-}^{13}\text{C}]$ retinal provided the $\text{C13} = \text{C14}$ and $\text{C15} = \text{N}$ configurations. From these data (Fig. 7A), the configuration of the AT and CS states, and the CS^* -, N-, and O-intermediates were determined as (13-*trans*, 15-*anti*), (13-*cis*, 15-*syn*), (13-*cis*, 15-*syn*), (13-*cis*, 15-*anti*), and (13-*trans*, 15-*anti*), respectively (Table 2). In situ photoirradiation solid-state NMR spectroscopy thus revealed the photoreaction pathways and structure of the AT and CS states, and the CS^* -, N-, and O-intermediates of the Y185F-bR mutant (Fig. 7B) (Oshima et al. 2015).

Conclusion

In situ photoirradiation solid-state NMR spectroscopy has been developed to allow the elucidation of photoreaction processes of retinal-binding photoreceptor membrane proteins.

In the case of SRII, the M-intermediates have a long half-life that allows the detection of these photointermediates in a stationary manner. Thus, M-, N', and O-intermediates were stationary trapped using in situ photoirradiation solid-state NMR spectroscopy at a lower temperature. It was also revealed that the M-intermediate transformed into the O-intermediate which then established equilibrium with the N'-intermediate by irradiation with UV light. The ^{13}C chemical shifts revealed that the M-, O-, and N'-intermediates have (13-*cis*, 15-*anti*), (13-*trans*, 15-*syn*), and (13-*cis*) configurations, respectively, with the aid of DFT calculations. In the case of SRI, M-intermediates accumulate by irradiation with green light to transmit attractant signals and P-intermediates accumulate by irradiation with UV light to transmit repellent signals in a color discriminating manner.

The photointermediates have a shorter half-life in the case of bR; therefore, they were trapped by freezing the state at a lower temperature and the NMR signals were observed by DNP methods. NMR signals of K-, L-, M-, N-, and O-intermediates were detected in the AT photocycle and that of the CS^* -intermediate was observed in the CS photocycle of Y185F-bR.

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Compliance with ethical standards

Conflict of interest Akira Naito declares that he has no conflict of interest. Yoshiteru Makino declares that he has no conflict of interest. Arisu Shigeta declares that she has no conflict of interest. Izuru Kawamura declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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