An Active Photoreceptor Intermediate Revealed by In Situ Photoirradiated Solid-State NMR Spectroscopy

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ABSTRACT A novel, to our knowledge, in situ photoirradiation system for solid-state NMR measurements is improved and demonstrated to successfully identify the M-photointermediate of *pharaonis* phoborhodopsin (*p*pR or sensory rhodopsin II), that of the complex with transducer (*p*pR/*p*HtrII), and T204A mutant embedded in a model membrane. 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 configuration coexisted under the continuously photoirradiated condition. NMR signals observed from the photoactivated retinal provide insights into the process of photocycle in the *p*pR/*p*HtrII complex.

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Pharaonis phoborhodopsin (*p*pR or sensory rhodopsin II) is a negative phototaxis receptor of *Natronomonas pharaonis,* and forms a 2:2 complex with the cognate transducer (*p*HtrII), which transmits the photosignal into cytoplasm (1,2). Light absorption of *p*pR initiates *trans-cis* photoisomerization of the retinal chromophore followed by cyclic chemical reaction consisting of several intermediates (K, L, M, and O) (3) as shown in Fig. 1.

K(540) state is an intermediate with the half-lifetime of 1 μs and has 13-*cis*, 15-*anti* retinal configuration. The L(488)-state can be transformed from the K-state with a half-lifetime of 30 μs and has 13-*cis*, 15-*anti* retinal configuration. After the L-state, proton is removed from the Schiff base group to form the M(390)-state with a long half-lifetime of 1.7 s and has 13-*cis*, 15-*anti* retinal configuration. Interestingly, a very long lifetime of the M-state has been reported with a half-lifetime of 2 h in a membrane mimetic containing *n*-octyl-β-D-glucoside (OG) (4). After the M-state, it transforms to the O(560)-state with a half-lifetime of 770 ms by taking protons and has 13-*trans*, 15-*syn* retinal configuration (5). Here, 540 nm, 488 nm, 390 nm, and 560 nm denote wavelengths of the maximum absorption, λ_{max} for K-, L-, M-, and O-intermediates, respectively. The M- and O-intermediates are thought to be active states for signal transduction. The crystal structure of the *p*pR/*p*HtrII complex suggests the formation of two specific hydrogen bonds between $Tyr^{199 ppR}$ and $Asn^{74 pHtrII}$, and between $Thr^{189 ppR}$ and $Glu^{43 pHtrII}$/$Ser^{62 pHtrII}$ (6). Thr^{204} is an important residue for color tuning and photocycle kinetics of *p*pR (7), and these observations provide additional important roles of Thr^{204} in *p*pR for the negative phototaxis function of the complex (8). Thus, T204A mutant does not show signal transduction activity despite forming M-intermediate.

Steric hindrance between C14-H of retinal and Thr^{204} occurred upon the formation of K-intermediate (9). At the same time, a specific hydrogen-bonding alteration occurred between Thr^{204} and Tyr^{174} in a *p*HtrII-dependent manner (10). Helix movement of *p*pR, outward tilting of the helix F, during the photocycle is suggested by various groups (11–13), and it is thought to be an essential step for the activation of *p*HtrII. However, no helix-tilting was observed in the crystal structure of the M-intermediate of the *p*pR/*p*HtrII complex (14). Thus, the structural changes upon the formation of the active M-intermediate continue to be an exciting topic of discussion. To this end, solid-state NMR techniques can be applied to elucidate site-specific positions for such membrane-embedded systems (15,16).

In this study, we successfully trapped the M-intermediate using the well-improved in situ photoirradiated solid-state NMR spectroscopy and photoirradiation efficiency was dramatically increased as compared with the previous system (17) (see Materials and Method in the Supporting Material). This system allowed us to observe the NMR signals of photointermediates to gain what we believe to be novel insights into the mechanism of signal transduction in the *p*pR/*p*HtrII complex.

[15-^{13}C, 20-^{13}C]retinal-*p*pR and *p*HtrII(1-159) with a His-Tag (6×His) at the C-terminal were expressed in the *Escherichia coli* BL21(DE3) strain in M9 medium. Purified proteins were incorporated into a lipid film of egg phosphatidylcholine (PC) (*p*pR/eggPC molar ratio of 1:30).

First, to investigate the trapping of an active intermediate of photoreceptor membrane proteins, we photoirradiated

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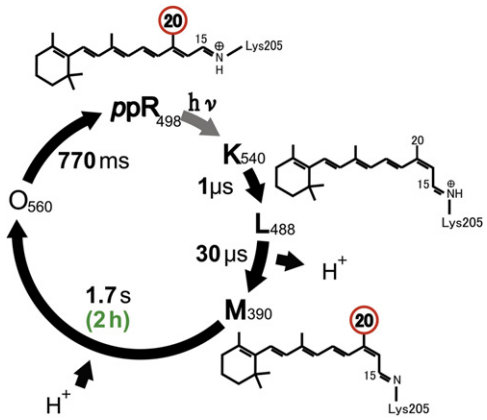

FIGURE 1 Photochemical reaction cycle of *pharaonis* phoborhodopsin (*p*pR). *p*pR absorbs blue light and forms K(540)-, L(488)-, M(390)-, and O(560)-intermediates. The M-intermediate has a long lifetime of 1.7 s in a membrane (3), whereas it has a much longer lifetime of 2 h in a membrane mimetic containing OG (4).

isomerization from the ground-state (G-state) to the M-intermediate of *p*pR, which was performed on [20-^{13}C] retinal-*p*pR dissolved in OG at 20°C. This is because the lifetime of the M-intermediate (2 h) is much longer than those of the other intermediates. The sample was light-irradiated outside the magnet and quickly inserted in the magnet to observe the ^{13}C directly detected magic-angle spinning (MAS) NMR spectrum of the M-intermediate (see Fig. S2 in the Supporting Material). The ^{13}C directly detected MAS NMR spectrum obtained from the G-state of *p*pR in OG showed a peak at 13.4 ppm. A new peak from the M-intermediate appeared at 21.6 ppm and the peak at 13.4 ppm completely disappeared. This observed chemical shift value is consistent with that of the M-intermediate of bacteriorhodopsin (BR) (18). This observation clearly showed that all-*trans* retinal in the G-state was efficiently transformed to the 13-*cis*, 15-*anti* configuration of retinal in the M-intermediate under the strong light-irradiation.

Second, in situ photoirradiated cross-polarization (CP) MAS solid-state NMR experiments were performed on a sample under near-physiological condition (in the lipid bilayer systems) such as [15-^{13}C, 20-^{13}C]retinal-*p*pR in egg PC at 0°C and −20°C (Fig. 2). In addition to the long lifetime for the M(390)-intermediate (1.7 s), the relatively long-lived O(560)-intermediate (0.77 s) can be irradiated by the light (532 nm). In this case, O-state is excited to G-state (3) and hence only M-intermediate can be trapped under the continuous photoirradiation condition. In the G-state, a peak of 20-^{13}C in retinal appeared at 13.3 ppm and the signal shifted to 22.3 ppm for the M-intermediate at 0°C (Fig. 2 *A*). The observation of peaks at 24.1, 22.5, and 21.7 ppm in *p*pR indicates the existence of at least three distinct M-intermediates under the photoirradiated condition at −20°C (Fig. 2 *C*). 15-^{13}C signals were also observed for light and dark conditions. It is noted that the signal position did not change between light and dark conditions,

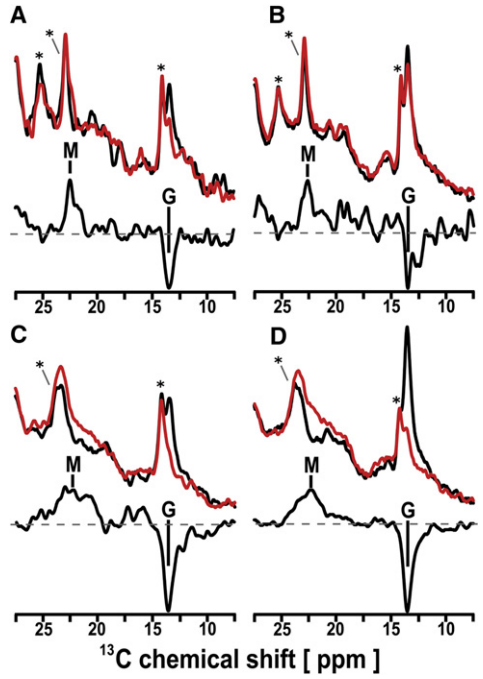

FIGURE 2 ^{13}C CP-MAS NMR spectra of [20-^{13}C, 15-^{13}C]retinal-*p*pR (*A* and *C*) and *p*pR/*p*HtrII complex (*B* and *D*) taken at 0°C (*A* and *B*) and −20°C (*C* and *D*) . (*Top spectra*) Data obtained from the light (*red*) and dark (*black*) states of *p*pR (*A* and *C*) and *p*pR/*p*HtrII complex (*B* and *D*). (*Bottom spectra*) Difference between those of light (*red*) and dark (*black*) spectra. The peaks M and G indicate the ^{13}C NMR signals of [20-^{13}C]retinal in the M-intermediate and the G-state, respectively. (*Asterisk*) Natural abundant lipid signals.

whereas intensity was reduced in the M-state (see Fig. S3). This observation is surprising because the shift of 5 ppm for 15-C from G (all-*trans*)- to M-state has been observed in BR (see Table S1 in the Supporting Material).

The yields of overall M-intermediates in *p*pR were evaluated to be 45% and 80% at 0 and −20°C, respectively, by inspecting the peak areas of the G-state. This difference in their yields can be attributed to a longer lifetime of the M-intermediate at a lower temperature. It is important to point out the single peak with a shoulder observed for the M-intermediate at 0°C, whereas multiplet lines were observed at −20°C. These multiplet signals can be attributed to the coexistence of several different interactions between the retinal and protein. The M-intermediates were also trapped for the [15-^{13}C, 20-^{13}C]retinal-*p*pR/*p*HtrII complex that has signal transduction function as revealed by the appearance of a CP MAS ^{13}C NMR peak at 22.6 ppm at 0°C (Fig. 2 *B*) and three distinct peaks at 23.5, 22.3, and 21.3 ppm at −20°C (see Table S1). Thus, multiple M-intermediates were also observed in the *p*pR/*p*HtrII complex at −20°C (Fig. 2 *D*). It was noted that the signal in the M-intermediate for *p*pR was slightly different from that in the *p*pR/*p*HtrII complex. The chemical shift values of [20-^{13}C]retinal in the M-intermediate of *p*pR distribute more widely and in a lower field than those of the *p*pR/*p*HtrII complex.

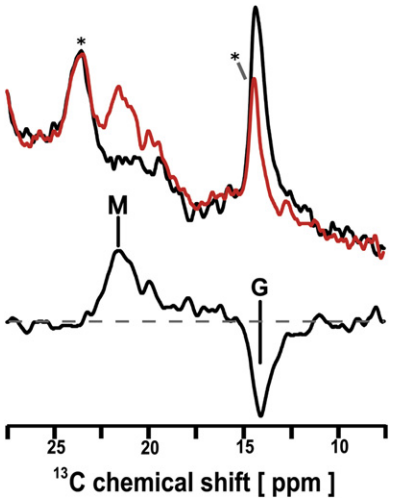

FIGURE 3 **^{13}C CP-MAS NMR spectra of [20-^{13}C] retinal-T204A taken at −20°C. (*Top spectra*) Data obtained from the light (*red*) and dark (*black*) states of T204A. (*Bottom spectrum*) Difference between those of light (*red*) and dark (*black*) spectra. The peaks M and G indicate the ^{13}C NMR signals of [20-^{13}C] retinal in the M-intermediate and the G-state, respectively. (*Asterisk*) Natural abundant lipid signals.**

Finally, the 20-^{13}C signal of T204A was observed as shown in Fig. 3. The signal in ground state at 14.0 ppm was converted to that in M-state at 21.3 ppm at −20°C. It is noted that the signal at 21 ppm did not show multiplet lines, but did show a singlet line. In addition, the chemical shift value of 20-^{13}C in the M-state is similar to that of BR rather than *p*pR. This result indicates that the M-state of T204A has 13-*cis*, 15-*anti* configuration and takes a single state.

In conclusion, we have successfully observed the M-intermediate from *p*pR, *p*pR/*p*HtrII complex, and T204A mutant embedded in a model membrane using in situ photoirradiated solid-state MAS NMR spectroscopy. Our results show that the multiple M-intermediates coexist at −20°C due to different retinal-protein interactions in *p*pR and also in the *p*pR/*p*HtrII complex. Multiple M-intermediates have been observed in BR as M_o and M_n states whose chemical shift difference was 1.8 ppm (19). Because only M-intermediate can be trapped, it is now possible to observe the structural changes of the protein side in the photoactivated state using in situ photoirradiated solid-state NMR experiments.

SUPPORTING MATERIAL

Materials and method, three figures, and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)01242-2.

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REFERENCES and FOOTNOTES

1. Spudich, J. L., and H. Luecke. 2002. Sensory rhodopsin II: functional insights from structure. *Curr. Opin. Struct. Biol.* 12:540–546.
2. Suzuki, D., H. Irieda, …, Y. Sudo. 2010. Phototactic and chemotactic signal transduction by transmembrane receptors and transducers in microorganisms. *Sensors (Basel Switzerland).* 10:4010–4039.
3. Roy, S., T. Kikukawa, …, N. Kamo. 2006. All-optical switching in *pharaonis* phoborhodopsin protein molecules. *IEEE Trans. Nanobioscience.* 5:178–187.
4. Sudo, Y., T. Nishihori, …, N. Kamo. 2008. A long-lived M-like state of phoborhodopsin that mimics the active state. *Biophys. J.* 95:753–760.
5. Imamoto, Y., Y. Shichida, …, T. Yoshizawa. 1992. Chromophore configuration of *pharaonis* phoborhodopsin and its isomerization on photon absorption. *Biochemistry.* 31:2523–2528.
6. Gordeliy, V. I., J. Labahn, …, M. Engelhard. 2002. Molecular basis of transmembrane signaling by sensory rhodopsin II-transducer complex. *Nature.* 419:484–487.
7. Shimono, K., T. Hayashi, …, N. Kamo. 2003. Importance of the broad regional interaction for spectral tuning in *Natronobacterium pharaonis* phoborhodopsin (sensory rhodopsin II). *J. Biol. Chem.* 278:23882–23889.
8. Sudo, Y., Y. Furutani, …, J. L. Spudich. 2006. Functional importance of the interhelical hydrogen bond between Thr^{204} and Tyr^{174} of sensory rhodopsin II and its alteration during the signaling process. *J. Biol. Chem.* 281:34239–34245.
9. Sudo, Y., Y. Furutani, …, H. Kandori. 2005. Steric constraint in the primary photoproduct of an archaeal rhodopsin from regiospecific perturbation of C-D stretching vibration of the retinyl chromophore. *J. Am. Chem. Soc.* 127:16036–16037.
10. Furutani, Y., K. Kamada, …, H. Kandori. 2005. Structural changes of the complex between *pharaonis* phoborhodopsin and its cognate transducer upon formation of the M photointermediate. *Biochemistry.* 44:2909–2915.
11. Wegener, A. A., I. Chizhov, …, H. J. Steinhoff. 2000. Time-resolved detection of transient movement of helix F in spin-labeled *pharaonis* sensory rhodopsin II. *J. Mol. Biol.* 30:881–891.
12. Spudich, J. L. 1998. Variations on a molecular switch: transport and sensory signaling by archaeal rhodopsins. *Mol. Microbiol.* 28:1051–1058.
13. Yoshida, H., Y. Sudo, …, N. Kamo. 2004. Transient movement of helix F revealed by photo-induced inactivation by reaction of a bulky SH-reagent to cysteine-introduced *pharaonis* phoborhodopsin (sensory rhodopsin II). *Photochem. Photobiol. Sci.* 3:537–542.
14. Moukhametzianov, R., J. P. Klare, …, V. I. Gordeliy. 2006. Development of the signal in sensory rhodopsin and its transfer to the cognate transducer. *Nature.* 440:115–119.
15. Kawamura, I., H. Yoshida, …, A. Naito. 2008. Dynamics change of phoborhodopsin and transducer by activation: study using D75N mutant of the receptor by site-directed solid-state ^{13}C NMR. *Photochem. Photobiol.* 84:921–930.
16. Etzkorn, M., K. Seidel, …, M. Baldus. 2010. Complex formation and light activation in membrane-embedded sensory rhodopsin II as seen by solid-state NMR spectroscopy. *Structure.* 18:293–300.
17. Kawamura, I., N. Kihara, …, A. Naito. 2007. Solid-state NMR studies of two backbone conformations at Tyr^{185} as a function of retinal configurations in the dark, light, and pressure adapted bacteriorhodopsins. *J. Am. Chem. Soc.* 129:1016–1017.
18. Bajaj, V. S., M. L. Mak-Jurkauskas, …, R. G. Griffin. 2009. Functional and shunt states of bacteriorhodopsin resolved by 250 GHz dynamic nuclear polarization-enhanced solid-state NMR. *Proc. Natl. Acad. Sci. USA.* 106:9244–9249.
19. Petkova, A. T., M. Hatanaka, …, J. Herzfeld. 2002. Tryptophan interactions in bacteriorhodopsin: a heteronuclear solid-state NMR study. *Biochemistry.* 41:2429–2437.