Advance Publication

Regular Article

Structure of a retinal chromophore of dark-adapted middle rhodopsin as studied by solid-state nuclear magnetic resonance spectroscopy

Izuru Kawamura^{1,2}, Hayato Seki¹, Seiya Tajima², Yoshiteru Makino^{1,5},
Arisu Shigeta¹, Takashi Okitsu³, Akimori Wada³, Akira Naito¹ and Yuki Sudo⁴

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

² Graduate School of Engineering and Science, Yokohama National University, Yokohama, Kanagawa 240-8501, Japan

³ Laboratory of Organic Chemistry for Life Science, Kobe Pharmaceutical University, Kobe, Hyogo 658-8558, Japan

⁴ Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

⁵ Present address: Graduate School of Medicine, Kobe University, Kobe, Hyogo 657-8501, Japan

doi: 10.2142/biophysico.bppb-v18.019

Received: March 18, 2021

Accepted: July 12, 2021

Released online in J-STAGE as advance publication: July 14, 2021

(Edited by Hideki Kandori)

Significance

Here, we analyzed the structure of the retinal chromophore of middle rhodopsin (MR) embedded in lipid bilayers using solid-state NMR spectroscopy. We recorded the ^{13}C NMR signals of three types of retinal configurations of dark-adapted MR exist: (13-*trans*, 15-*anti*), (13-*cis*, 15-*syn*), and 11-*cis* isomers. Furthermore, we discretely observed $^{13}\text{C}\zeta$ NMR signals of Tyr185 in MR with the all-*trans* and 13-*cis* isomers. The ^{15}N NMR signal of the protonated Schiff base in MR exhibited a strong electrostatic interaction with the counter ion. Our study makes a significant contribution to the literature because we highlighted the key structural chromophores of MR.

**Structure of a retinal chromophore of dark-adapted middle rhodopsin
as studied by solid-state nuclear magnetic resonance spectroscopy**

Izuru Kawamura,^{†*} Hayato Seki,[†] Seiya Tajima[#], Yoshiteru Makino,^{†\$} Arisu Shigeta,[†]
Takashi Okitsu,[‡] Akimori Wada,[‡] Akira Naito,[†] Yuki Sudo[∂]

Running title: Structure of a retinal chromophore of rhodopsin

[†]Graduate School of Engineering, Yokohama National University, Hodogaya-ku, Yokohama 240-8501, Japan,

[#]Graduate School of Engineering and Science, Yokohama National University, Hodogaya-ku, Yokohama 240-8501, Japan,

[‡]Laboratory of Organic Chemistry for Life Science, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan

[∂]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan.

*Corresponding author: izuruk@ynu.ac.jp

\$Present address: Graduate School of Medicine, Kobe University, Kobe 657-8501, Japan

19

20 **ABSTRACT**

21 Middle rhodopsin (MR) found from the archaeon *Haloquadratum walsbyi* is
22 evolutionarily located between two different types of rhodopsins, bacteriorhodopsin (BR)
23 and sensory rhodopsin II (SRII). Some isomers of the chromophore retinal and the
24 photochemical reaction of MR are markedly different from those of BR and SRII. In this
25 study, to obtain the structural information regarding its active center (i.e., retinal), we
26 subjected MR embedded in lipid bilayers to solid-state magic-angle spinning nuclear
27 magnetic resonance (NMR) spectroscopy. The analysis of the isotropic ^{13}C chemical
28 shifts of the retinal chromophore revealed the presence of three types of retinal
29 configurations of dark-adapted MR: (13-*trans*, 15-*anti* (all-*trans*)), (13-*cis*, 15-*syn*), and
30 11-*cis* isomers. The higher field resonance of the 20-C methyl carbon in the all-*trans*
31 retinal suggested that Trp182 in MR has an orientation that is different from that which it
32 shows in other microbial rhodopsins, owing to the changes in steric hindrance associated
33 with the 20-C methyl group in retinal. ^{13}C signals of Tyr185 in MR for all-*trans* and 13-
34 *cis*, 15-*syn* isomers were discretely observed, representing the difference in the hydrogen
35 bond strength of Tyr185. Further, ^{15}N NMR analysis of the protonated Schiff base
36 corresponding to the all-*trans* and 13-*cis*, 15-*syn* isomers in MR showed a strong

37 electrostatic interaction with the counter ion. Therefore, the resulting structural
38 information exhibited the property of stable retinal conformations of dark-adapted MR.
39 **Keywords:** microbial rhodopsin; retinal isomers; nuclear magnetic resonance; protonated
40 Schiff base; chemical shifts

41 **Introduction**

42 Microbial rhodopsins have been identified in microorganisms, including
43 haloarchaea and halobacteria, and have seven transmembrane helices with all-*trans*
44 retinal chromophores via a protonated Schiff base (PSB) [1,2,3]. In the family of
45 microbial rhodopsins, novel types of rhodopsins are being identified, despite the similar
46 basic structural motif (7 transmembrane helices + retinal chromophore), which are used
47 as tools in optogenetics [1,2,3]. Several types of microbial rhodopsins function as light-
48 driven ion pumps, such as proton pump bacteriorhodopsin (BR) and proteorhodopsin
49 (PR), chloride pump halorhodopsin (HR), and sodium ion pump rhodopsin (NaR)
50 [4,5,6,7]. The light-driven divalent sulfate ion transporter rhodopsin (SyHR) has recently
51 been identified [8]. The other type involves photoreceptors, such as sensory rhodopsin I
52 (SRI) and II (SRII) for positive and negative phototaxis responses, respectively [9,10,11].
53 *Anabaena* sensory rhodopsin (ASR), with its soluble transducer from cyanobacteria,
54 regulates the expression of photosynthesis-related genes [12]. In addition, functional and
55 color conversions have been achieved by crucial mutations in genes encoding amino acid
56 residues near the retinal chromophore [13,14,15]. Therefore, the structure of the retinal-
57 binding site plays an important role in understanding its function.

58 Middle rhodopsin (MR), isolated from the square-shaped haloarchaeon
 59 *Haloquadratum walsbyi*, is evolutionarily located between BR and SRII [16,17]. The
 60 structure of the retinal binding pocket in BR is shown with the corresponding residues of
 61 MR in Figure 1. MR exhibits a larger blue-shifted absorption at 485 nm than BR. Dark-
 62 adapted MR in detergent micelles and phosphatidylglycerol (PG) liposomes has three
 63 retinal isomers, as revealed by extraction of retinal and HPLC analysis: all-*trans* (36.5%),
 64 13-*cis* (56.4%), and 11-*cis* (7.6%) in micelles and all-*trans* (58.2%), 13-*cis* (38.9%), and
 65 11-*cis* (2.9%) in liposomes [16,18]. The individual absorption maxima and independent
 66 photochemical reactions corresponding to these three isomers have also been
 67 demonstrated via UV-Vis and flash photolysis experiments [19]. Notably, MR is the only
 68 microbial rhodopsin with the 11-*cis* retinal as a chromophore like animal rhodopsins.
 69 Both K and M intermediates of MR appear only in the photocycle of all-*trans* isomers.
 70 The production of these intermediates is one of the key functional events among microbial
 71 rhodopsins. Therefore, it is expected that the photocycle of all-*trans* isomers through K
 72 and M-intermediates is functionally important. However, the type of function in MR is
 73 still unknown. MR does not show H⁺ pump activity even though it establishes a BR-like
 74 fast photocycle [16]. Additionally, regarding *Np*SRII in *Natronomonas pharaonis*,
 75 Thr189 and Tyr199 are used to form a complex with its cognate transducer protein, while

76 Tyr174 and Thr204 play an essential role in the expression of negative phototaxis
77 [13,20,21]. The complex of the A201T/M211Y MR mutant (with residues corresponding
78 to those of *Np*SRII; i.e., Tyr185, Thr201, Tyr211, and Thr216) with the transducer protein
79 HtrII shows phototaxis response to >460 nm light; however wild-type MR does not [16].
80 Therefore, it is important to consider that among microbial rhodopsins, MR has unique
81 properties and clarify why the dark-adapted MR shows extremely short wavelength light
82 absorption and has multiple retinal isomers.

83 Solid-state nuclear magnetic resonance (NMR) spectroscopy, which offers the
84 possibility to observe not only the structure and dynamics of proteins in cell membranes,
85 but also photo intermediates by isomerization of retinal, has already been applied in
86 structural studies of several rhodopsins [22-26].

87 Therefore, in this study, we investigated the structural characteristics of the
88 retinal chromophore in MR via ^{13}C and ^{15}N solid-state NMR spectroscopy to the end of
89 elucidating the structure of dark-adapted MR, which has multiple retinal isomers.

90

91 **MATERIALS AND METHODS**

92 **Expression and reconstitution of MR into lipid bilayers**

MR was overexpressed in *Escherichia coli* C41 (DE3) cells. The bacteria were grown in M9 medium containing 50 mg of [$^{13}\text{C}\zeta$]Tyr and [$^{15}\text{N}\zeta$]Lys (Cambridge Isotope Laboratories, Andover, MA, USA) per liter at 37°C until an OD₆₆₀ of 0.8 was reached. After adding 1 mM isopropyl thiogalactoside (IPTG) and 5 μM [14, 20- ^{13}C]-labeled all-*trans* retinal, protein expression of the protein was performed at 25 °C for 15 h followed by cell lysis via ultrasonication, after which the protein was solubilized using *n*-dodecyl- β -D-maltoside (DDM). The recombinant MR with a C-terminal His-tag was purified using nickel-nitrilotriacetic acid agarose. Thereafter, the MR was reconstituted into an L- α -phosphatidyl-DL-glycerol (Egg-PG) membrane at a molar ratio of 1:30 at pH 7.0.

103 **Solid-state NMR experiments**

The reconstituted sample (approximately 8.0 mg of protein) was concentrated via centrifugation and packed into the central part of a 4.0-mm diameter zirconia rotor under dim light. The rotor was maintained in the dark for two days. ^{13}C and ^{15}N solid-state NMR experiments were performed on a 600 MHz Bruker Avance III spectrometer equipped with a ^1H -X double-resonance probe. Further, ^{13}C and ^{15}N cross-polarization and magic-angle spinning (CP-MAS) experiments were performed at a setting temperature of 10°C under a MAS frequency of 9.3 kHz, while ^{13}C - ^{13}C dipolar-assisted

111 rotational resonance (DARR) [27] experiments were performed at a mixing time of 500
112 ms with 64 and 2048 acquisition points in the f_1 and f_2 dimensions. A spinal-64 proton
113 high-power decoupling field of 78 kHz was employed during each acquisition [28]. ^{13}C
114 chemical shifts were externally referenced to the methylene resonance of adamantane at
115 40.48 ppm (trimethylsilylpropanesulfonate (DSS) at 0.0 ppm), while ^{15}N chemical shifts
116 were externally referenced to the $^{15}\text{NH}_4\text{Cl}$ crystal at 38.34 ppm. The acquired NMR data
117 were phased and baseline-corrected using the TOPSPIN software. A deconvolution
118 procedure was applied to the ^{15}N CP-MAS spectrum to obtain the values of chemical
119 shift, line width, and signal intensity values in the spectral region of PSB. Furthermore,
120 two peaks were used to reproduce the experimental spectra corresponding to all-*trans* and
121 13-*cis* isomers. The best-fitting results were obtained based on the Gaussian shapes
122 corresponding to two peaks using Bruker TOPSPIN software.

123

124 **Results and Discussion**

125 **^{13}C NMR signals of retinal isomers in MR**

126 In the MR reconstituted into PG liposome, the main retinal configurations in the
127 dark were all-*trans* and 13-*cis* forms as observed via HPLC [18]. First, we performed an
128 *in-situ* analysis of the composition of the retinal isomers of dark-adapted MR embedded

129 in PG liposome using ^{13}C solid-state NMR experiments. As shown in Figure 2 (a), the
 130 comparison of the ^{13}C CP-MAS NMR spectra corresponding to stable isotope-labeled and
 131 non-labeled MR samples showed that $[14, 20\text{-}^{13}\text{C}]$ retinal and $[^{13}\text{C}\zeta]$ Tyr NMR signals
 132 could be distinguished from the ^{13}C NMR signals corresponding to naturally abundant
 133 proteins and lipids. Further, using the extensive ^{13}C NMR data [22, 29-31] obtained for
 134 the structure of retinal in rhodopsin proteins and retinal compounds summarized in
 135 Supplementary Table S1, we identified the following chemical shift values for the 13-
 136 *trans*, 15-*anti* (all-*trans*) isomer in MR: $14\text{-}^{13}\text{C}$: $\delta = 124.5$ ppm (Figure 2(b)) and $20\text{-}^{13}\text{C}$:
 137 $\delta = 14.6$ ppm (Figure 2(c)). Although the NMR signal of $14\text{-}^{13}\text{C}$ and $20\text{-}^{13}\text{C}$ in the 13-*cis*
 138 form appeared at approximately 116 ppm and 23.0 ppm, respectively, the signal intensity
 139 was relatively low (Figure 2(b) and (c)). Reportedly, a $20\text{-}^{13}\text{C}$ signal of an 11-*cis* retinal
 140 compound in C_6D_6 has been observed at approximately 17–19 ppm [31]. The signal of
 141 the 11-*cis* form with an extremely low intensity was observed at 19 ppm (Figure 2 (c)).
 142 Thus, we estimated the fractions of the three retinal isomers from the individual peak
 143 areas of $20\text{-}^{13}\text{C}$ based on the differences in the spectra corresponding to stable-isotope
 144 labeled and non-labeled MR (Figure 2(a), black and red, respectively) as all-*trans*,
 145 ~72.9%; 13-*cis*, ~19.7%; and 11-*cis*, ~7.4%. For this reason, we suggested that the all-
 146 *trans* isomer in the dark-adapted MR was predominant in the PG membrane. Thus, the

20-¹³C signal of the all-*trans* isomer in MR appeared at 14.6 ppm and showed higher field shift compared with those of BR at 15.2 ppm, *Np*SRII at 15.3 ppm [32], and SRI at 15.8 ppm [33] (as a reference of ¹³C NMR to DSS). The all-*trans* retinal in BR has a steric hindrance with Trp182, as well as a short inter-atomic distance of 3.35 Å between the 20-methyl carbon and indole nitrogen of Trp182 based on solid-state NMR data and crystal structural information (Figure 1) [34,35]. Further, the comparison of the UV resonance Raman spectra of wild-type BR and the W182F mutant showed a strong interaction between the indole ring of Trp182 and both 19-C and 20-C of retinal [36]. Trp182 in BR plays an important role in re-isomerization from 13-*cis* to all-*trans* in the later stage of the photocycle [37]. The higher field resonance of the 20-C methyl carbon in the all-*trans* retinal observed in this study suggested that in MR, Trp182 has an orientation that is different from that which it shows in other microbial rhodopsins, owing to the changes in steric hindrance associated with the 20-C methyl group in retinal. Particularly, the chemical shift value of 20-C in MR was clearly different from that of *Np*SRII, which has almost the same maximum absorption wavelength and only the all-*trans* isomer. Additionally, considering microbial rhodopsins, it is possible that a rearrangement of a water molecule hydrogen bonded to the indole ring of conserved Trp (Trp182 in BR) occurred in MR. Therefore, the unusual 20-C NMR signal of MR possibly suggested that

the interaction of 20-C methyl groups in the retinal with Trp182 is related to MR having multiple isomers of MR.

Assessment of the Tyr185 NMR signals assigned by DARR

Figure 3 shows the ^{13}C DARR spectra of [14, 20- ^{13}C] retinal and [$^{13}\text{C}\zeta$] Tyr-labeled MR in the dark. From this figure, it is evident that the NMR signals of the methyl carbon at the 20- ^{13}C of retinal as well as that of the carbon at the 14- ^{13}C position showed two cross peaks with the $\text{C}\zeta$ of Tyr: 20- ^{13}C in all-*trans*/ $^{13}\text{C}\zeta$ Tyr at 14.6 / 159.5 ppm, and 20- ^{13}C in 13-*cis*, 15-*syn*/ $^{13}\text{C}\zeta$ Tyr at 23.2 / 159.0 ppm. As a reference for the retinal-binding site of BR with the corresponding residues of MR in Figure 1, the correlated Tyr NMR signal could be assigned to Tyr185 in MR, which is closest to retinal. Interestingly, the chemical shift values of $\text{C}\zeta$ in Tyr185 differed slightly, between two conformations, and were thus assigned to the all-*trans* and 13-*cis*/15-*syn* configurations. Further, the structure of the retinal-binding site could be altered depending on the retinal configurations in the dark-adapted MR. Such structural changes in specific residues corresponding to retinal isomers in the dark adaptation have been similarly reported as simultaneous observations of the two signals of Tyr185 in dark-adapted BR [38]. Further, it has been reported that the chemical shift value of Tyr $\text{C}\zeta$ is sensitive to hydrogen bond formation [23,39,40]. The

183 Tyr185 NMR signal corresponding to the all-*trans* isomer of MR at 159.5 ppm was
184 similar to that of Tyr185 in BR (160.0 ppm) and Tyr174 in *NpSR*II (159.7 ppm) [41,42].
185 In contrast, the signal of Tyr179 in ASR showed a lower chemical shift at 156.4 ppm
186 given that ASR has no corresponding Asp residue, rather has Pro206, which does not
187 form hydrogen bonds [26]. Thus, the value of $^{13}\text{C}\zeta$ Tyr185 indicated that Tyr185 in MR,
188 at least, forms a hydrogen bond. It also suggested that Tyr185 in MR can interact with
189 Asp213 or Thr216 via hydrogen bonding, e.g., Tyr174-Thr204 in *NpSR*II [43]. The
190 conserved Tyr is an essential residue in the reaction center of microbial rhodopsins given
191 that its mutants have a significant effect on the efficacy of the light-driven proton pump
192 in BR or the light-induced signal transduction in *NpSR*II [11, 44]. Further, given
193 importance of the formation of the Tyr185-Thr215 hydrogen bond in the signal transduce-
194 able BR mutant with respect to the expression of negative phototaxis [20], the hydrogen
195 bond between Tyr185 and Thr216 in MR plays an essential role in the signal transduce-
196 able mutant of MR [16]. In the case of *NpSR*II, it has been reported that the surrounding
197 residues around the retinal chromophore largely contribute to the color tuning of the
198 protein [45]. Furthermore, the Tyr-retinal interactions in MR may be related to the slight
199 differences in the maximal absorption wavelength (λ_{max}) of 485 nm (all-*trans*) and 479
200 nm (13-*cis*, 15-*syn*) [19]. Unfortunately, the cross peak between 20-C and 14-C from 11-

cis was not identified owing to the extremely low population of this isomer. Therefore, our results suggested that Tyr185 is one of the residues that controls the $\lambda_{\max}$ as well as the proportion of the retinal conformation in dark-adapted MR.

¹⁵N NMR signal of the PSB

In MR, the retinal chromophore is covalently bonded to Lys217 via a PSB linkage. In the ¹⁵N CP-MAS NMR spectrum of [¹⁵N]Lys-labeled dark-adapted MR, NMR signals corresponding to the free Lys side chain, Arg side chain, backbone amide nitrogen, and PSB were identified, as shown in Figure 4 (a). The PSB signal appeared at approximately 184 ppm, and the two peak components of PSB were simply estimated as an intensity ratio of 3.5 (at 184.5 ppm) : 1 (at 181.0 ppm) based on spectral deconvolution analysis, as shown in Figure 4 (b). Consequently, we suggested that the PSB signals in dark-adapted MR at 184.5 ppm could be attributed to the all-*trans* form, while that at 181.0 ppm could be attributed to the 13-*cis*, 15-*syn* form based on the ¹³C NMR intensity of the retinal isomers. The deconvolution result was ignored as a slight contribution from the 11-*cis* isomer. Based on a previous report on the all-*trans* and 13-*cis* isomer PSB-counter ion model complexes, a linear relationship in the range of 20 ppm or more exists between the ¹⁵N chemical shift of PSB and the $\lambda_{\max}$ depending on the halide ion species (Figure 5

(a) and (b)) [46,47]. MR showed the shortest $\lambda_{\max}$ among the microbial rhodopsin families. Specifically, the $\lambda_{\max}$ values corresponding to the three retinal configurations of MR were distinguished as 485 nm (*all-trans*), 479 nm (13-*cis*, 15-*syn*), and 495 nm (11-*cis*) by the extraction of single component UV-Vis spectra [19]. As a reference for the maximum MR data, the value for MR was plotted together with those for BR [48], *Krokinobacter* rhodopsin 2 (KR2) [40], ASR [49], *Thermophilic* rhodopsin (TR) [50], and *NpSR*II [51] as shown in Figure 5 (a) and (b). MR with the *all-trans* form (485 nm / 184.5 ppm) agreed well with the trend of the linear relationships shown in the model compound (Figure 5 (a)). Compared with the values of other rhodopsins, MR had a lower field resonance of PSB with a shorter $\lambda_{\max}$. Further, MR with the 13-*cis*, 15-*syn* form (479 nm / 181.0 ppm) also showed agreement with the trend, but deviated more strongly from the model behavior than BR with the 13-*cis*, 15-*syn* form as shown in Figure 5 (b). It was also observed that PSB in both the *all-trans* and 13-*cis* retinal isomers of MR exhibited a strong electrostatic interaction with the counter ion. The most promising counter ion candidates were Asp84 in helix C and Asp213 in helix G. Possibly, the relative positions of PSB and the carboxyl groups (COO⁻) of Asp84 and Asp213 are closer than those in the other microbial rhodopsins. During the *all-trans* photocycle, structural changes in the β -sheet occur in the hydrophilic part of the protein, suggesting an extracellular BC loop, as

observed via time-resolved FT-IR spectroscopy [18]. Quantum mechanics/molecular mechanics (QM/MM) calculations involving *NpSR*II have suggested that the $\lambda_{\max}$ at 498 nm is induced by a displacement of helix G, resulting in the observed distance between PSB and Asp201 [52]. Thus, our ^{15}N NMR result suggested that the strong electrostatic interaction between PSB and Asp84 in helix C or Asp213 in helix G is significantly linked to the shorter $\lambda_{\max}$ of the all-*trans* form in MR. In the case of the *NpSR*II D75E mutant, the population of 13-*cis* species in the dark increased as the proportion of 13-*cis* to all-*trans* became 6:4, given that it may be closer to the relative interatomic distance between PSB and the counter ion Glu75 [53]. Thus, it could be suggested that MR is capable of forming a stable 13-*cis* isomer based on electrostatic interactions with Asp84 or Asp213 as well as the contacts between retinal and surrounding residues containing the above-mentioned interactions of 20-C methyl groups with Trp182. Additionally, the deviation of the value corresponding to the 13-*cis*, 15-*syn* form of MR from the trend of the model compound could be attributed to the tilt around the C=N and N-C ϵ (Lys217) bonds, which are influenced by closer counter ions or surrounding residues (Figure 5(b)). The cause of the formation of the 11-*cis* form in MR, which is a minor conformation, is unknown, but it can be suggested that the structure of the 11-*cis* retinal isomer is also regulated by the interactions between retinal and the surrounding residues.

255

256 **Conclusions**

257 In this study, we applied solid-state NMR analysis to dark-adapted MR in a PG membrane.

258 The structures of the three retinal isomers in MR were determined from the results of the

259 ^{13}C solid-state NMR. We recorded the higher field resonance of 20- ^{13}C NMR signal of260 *all-trans* retinal in MR compared with that of other microbial rhodopsins, suggesting the261 difference in the interaction of 20-C methyl group with Trp182. The *all-trans* and 13-*cis*,262 15-*syn* isomers showed a difference in the hydrogen bond strength of Tyr185. Further,263 the ^{15}N signal of PSB revealed the existence of strong electrostatic interactions with the264 counter ion for *all-trans* and 13-*cis*, 15-*syn* isomers. Based on the structural information

265 obtained via solid-state NMR, we suggested that the characteristic interactions at the

266 retinal binding site affects the retinal isomers of the dark-adapted MR. Therefore, solid-

267 state NMR spectroscopy is a promising tool for studying the structures of several

268 coexisting retinal isomers as well as the interactions of PSB in microbial rhodopsin.

269

270 **Acknowledgments**

271 This work was supported by Grants-in-Aid for Scientific Research in an Innovative Area

272 KAKENHI (JP16H06043, JP26104513 and JP20H05211 to I.K., JP25104005,

JP25104009 and JP19H05396 to Y.S.) from the Ministry of Culture, Sports, Science and Technology, Japan (MEXT), and Grants-in-Aid for Scientific Research (B) (JP18H02387 to I.K and JP18H02411 to Y.S.) and Scientific Research (C) (JP15K06963 to A. N.) from the Japan Society for the Promotion of Science (JSPS).

Conflicts of Interest

The authors declare that no competing interests exist.

Author contribution

I.K., A.N., and Y.S. directed the project. I.K. wrote the paper. H.S. expressed the protein and prepared a solid-state NMR sample. I. K., H.S., and Y.M. performed the solid-state NMR measurements. I.K., S.T., Y.M., and A.S. analyzed the ^{13}C and ^{15}N NMR data. T.O. and A.W. synthesized the ^{13}C stable isotope-labeled retinal.

References

- Ernst, O. P., Lodowski, D. T., Elstner, M., Hegemann, P., Brown L. S., & Kandori, H. Microbial and animal rhodopsins: structures, functions, and molecular mechanisms. *Chem. Rev.* **114**, 126-163 (2014). DOI: 10.1021/cr4003769

- 291 2. Kurihara M. & Sudo, Y. Microbial rhodopsins: wide distribution, rich diversity and great
292 potential. *Biophys. Psychobiol.* **12**, 121-129 (2015). DOI:
293 10.2142/biophysico.12.0_121
- 294 3. Kojima, K., Shibukawa, A., & Sudo, Y. The unlimited potential of microbial rhodopsins
295 as optical tools. *Biochemistry* **59**, 218-229 (2020). DOI: 10.1021/acs.biochem.9b00768
- 296 4. Subramaniam, S. & Henderson, R. Molecular mechanism of vectorial proton
297 translocation by bacteriorhodopsin. *Nature* **406**, 653-657 (2000). DOI:
298 10.1038/35020614
- 299 5. Giovannoni, S. J., Bibbs, L., Cho, J. C., Stepels, M. D., Desiderio, R., Vergin, K. L., *et*
300 *al.* Proteorhodopsin in the ubiquitous marine bacterium SAR11. *Nature* 438, 82-85
301 (2005). DOI: 10.1038/nature04032
- 302 6. Ruediger, M. & Oesterhelt, D. Specific arginine and threonine residues control anion
303 binding and transport in the light-driven chloride pump halorhodopsin. *EMBO J.* **16**,
304 3813-3821 (1997). DOI: 10.1093/emboj/16.13.3813
- 305 7. Inoue, K., Ono, H., Abe-Yoshizumi, R., Yoshizawa, S., Ito, H., Kogure, K., *et al.* A light-
306 driven sodium ion pump in marine bacteria. *Nat. Commun.* **4**, 1678 (2013). DOI:
307 10.1038/ncomms2689
- 308 8. Niho, A., Yoshizawa, S., Tsukamoto, T., Kurihara, M., Tahara, S., Nakajima, Y., *et al.*

- 309 Demonstration of a light-driven SO_4^{2-} transporter and its spectroscopic characteristics. *J.*
 310 *Am. Chem. Soc.* **139**, 4376-4389 (2017). DOI: 10.1021/jacs.6b12139
- 311 9. Spudich, J. L., Yang, C. S., Jung K. H., & Spudich, E. N. Retinylidene proteins: Structures
 312 and functions from archaea to humans. *Annu. Rev. Cell. Biol.* **16**, 365-392 (2000). DOI:
 313 10.1146/annurev.cellbio.16.1.365
- 314 10. Spudich, J. L., & Luecke, H. Sensory rhodopsin II: functional insights from structure.
 315 *Curr. Opin. Struct. Biol.* **12**, 540-546 (2002). DOI: 10.1016/s0959-440x(02)00359-7
- 316 11. Sudo, Y., Kandori, H., & Kamo, N. Molecular mechanism of protein-protein interaction
 317 of pharaonis phoborhodopsin/ transducer and photo-signal transfer reaction by the
 318 complex. *Recent Res. Devel. Biophys.* **3**, 1-16 (2004).
- 319 12. Jung, K. H., Trivedi, V. D., & Spudich, J. L. Demonstration of a sensory rhodopsin in
 320 eubacteria. *Mol. Microbiol.* **47**, 1513-1522 (2003). DOI: 10.1046/j.1365-
 321 2958.2003.03395.x
- 322 13. Sudo, Y., Furutani, Y., Kandori, H., & Spudich, J. L. Functional importance of the
 323 interhelical hydrogen bond between Thr204 and Tyr174 of sensory rhodopsin II and its
 324 alteration during the signaling process. *J. Biol. Chem.* **281**, 34239-34245 (2006). DOI:
 325 10.1074/jbc.m605907200
- 326 14. Sasaki, J., Brown, L. S., Chon, Y. S., Kandori, H., Maeda, A., Needleman, R., *et al.*

- 327 Conversion of bacteriorhodopsin into a chloride ion pump. *Science* **269**, 73-75 (1995).
328 DOI: 10.1126/science.7604281
- 329 15. Kato, H. E., Kamiya, M., Sugo, S., Ito, J., Taniguchi, R., Orito, A., *et al.* Atomistic design
330 of microbial opsin-based blue-shifted optogenetics tools. *Nat. Commun.* **6**, 7177 (2015).
331 DOI: 10.1038/ncomms8177
- 332 16. Sudo, Y., Ihara, K., Kobayashi, S., Suzuki, D., Irieda, H., Kikukawa, T., *et al.* A microbial
333 rhodopsin with a unique retinal composition shows both sensory rhodopsin II and
334 bacteriorhodopsin-like properties. *J. Biol. Chem.* **286**, 5967-5976 (2011). DOI:
335 10.1074/jbc.m110.190058
- 336 17. Inoue, K., Tsukamoto, T., & Sudo, Y. Molecular and evolutionary aspects of microbial
337 sensory rhodopsins. *Biochim. Biophys. Acta Bioenerg.* **1837**, 562-577 (2014). DOI:
338 10.1016/j.bbabbio.2013.05.005
- 339 18. Furutani, Y., Okitsu, T., Reissig, L., Mizuno, M., Homma, M., Wada, A., *et al.* Large
340 spectral change due to amide modes of a β -sheet upon the formation of an early
341 photointermediate of middle rhodopsin. *J. Phys. Chem. B* **117**, 3449-3458 (2013). DOI:
342 10.1021/jp308765t
- 343 19. Inoue, K., Reissig, L., Sakai, M., Kobayashi, S., Homma, M., Fujii, M., *et al.* Absorption
344 spectra and photochemical reactions in a unique photoactive protein, middle rhodopsin

- 345 MR. *J. Phys. Chem. B.* **116**, 5888-5899 (2012). DOI: 10.1021/jp302357m
- 346 20. Sudo, Y. & Spudich, J. L. Three strategically placed hydrogen-bonding residues convert
347 a proton pump into a sensory receptor. *Proc. Natl. Acad. Sci. U. S. A.*, **103**, 16129-16134
348 (2016). DOI: 10.1073/pnas.0607467103
- 349 21. Sudo, Y., Yamabi, M., Kato, S., Hasegawa, C., Iwamoto, M., Shimono, K., *et al.*
350 Importance of specific hydrogen bonds of archaeal rhodopsins for the binding to the
351 transducer protein. *J. Mol. Biol.* **357**, 1274-1282 (2006). DOI: 10.1016/j.jmb.2006.01.061
- 352 22. Naito, A., Makino, Y., Shigeta, A., Kawamura, I. Photo-reaction pathways and photo
353 intermediates of retinal-binding photo-receptor proteins as revealed by *in-situ* photo-
354 irradiation solid-state NMR spectroscopy. *Biophys. Rev.* , **11** (2), 167-181 (2019). DOI:
355 10.1007/s12551-019-00501-w
- 356 23. Kimata, N., Pope, A., Eilers, M., Opefi, C.A., Zilx, M., Hirshfeld, A., *et al.* Retinal
357 orientation and interactions in rhodopsin reveal a two-stage trigger mechanism for
358 activation. *Nat. Commun.* **7**, 12683, (2016). DOI: 10.1038/ncomms12683
- 359 24. Hatcher, M. E., Hu, J. G., Belenky, M., Verdegem, P., Lugteburg, J., Griffin, R. G., *et*
360 *al.* Control of the pump cycle in bacteriorhodopsin: mechanisms elucidated by solid-state
361 NMR of the D85N mutant. *Biophys. J.* **82**, 1017-1029 (2002). DOI: 10.1016/s0006-
362 3495(02)75461-1

- 363 25. Becker-Baldus, J., Bamann, C., Saxena, K., Gustmann, H., Brown, L. J., Brown, R. C.
364 D., *et al.* Enlightening the photoactive site of channelrhodopsin-2 by DNP-enhanced
365 solid-state NMR spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 9896-9011 (2015).
366 DOI: 10.1073/pnas.1507713112
- 367 26. Wang, S., Munro, R. A., Shi, L., Kawamura, I., Okitsu, T., Wada, A., *et al.* Solid-State
368 NMR Structure of a Lipid-Embedded Heptahelical Membrane Protein Trimer. *Nature*
369 *Methods* **10** (10), 1007-1012 (2013). DOI:10.1038/NMETH.2635
- 370 27. Takegoshi, K., Nakamura, S., & Terao, T. ^{13}C - ^1H dipolar-assisted rotational resonance
371 in magic-angle spinning NMR. *Chem. Phys. Lett.* **344**, 631-637 (2011). DOI:
372 10.1016/s0009-2614(01)00791-6
- 373 28. Fung, B. M., Khitritin, A. K., & Emolaev, K. An improved broadband decoupling sequence
374 for liquid crystals and solids. *J. Magn. Reson.* **142**, 97-101 (2000). DOI:
375 10.1006/jmre.1999.1896
- 376 29. Ding, X., Zhao, X., & Watts, A. G-protein-coupled receptor structure, ligand binding and
377 activation as studied by solid-state NMR spectroscopy. *Biochem. J.* **450**, 443-457 (2013).
378 DOI: 10.1042/bj20121644
- 379 30. Laksmi, K. V., Farrar, M. R., Raap, J., Lugtenburg, J., Griffin, R. G., & Herzfeld, J. Solid
380 state ^{13}C and ^{15}N NMR investigations of the N intermediate of bacteriorhodopsin.

- 381 *Biochemistry* **33**, 8853-8857 (1994). DOI: 10.1021/bi00196a001
- 382 31. Wang, K. W., Wang, S. W., & Du, Q. Z. Complete NMR assignment of retinal and its
383 related compounds. *Magn. Reson. Chem.* **51**, 435-438 (2013). DOI: 10.1002/mrc.3956
- 384 32. Makino, Y., Kawamura, I., Okitsu, T., Wada, A., Kamo, N., Sudo, Y., *et al.* Retinal
385 configuration of ppR intermediates revealed by photoirradiation solid-state NMR
386 and DFT. *Biophys. J.* **115**, 72-83 (2018). DOI: 10.1016/j.bpj.2018.05.030
- 387 33. Yomoda, H., Makino, Y., Tomonaga, Y., Hidaka, T., Kawamura, I., Okitsu, T., *et al.*
388 Color-discriminating retinal configurations of sensory rhodopsin I by photo-irradiation
389 solid-state NMR spectroscopy. *Angew. Chem. Int. Ed.* **53**, 6960-6964 (2014). DOI:
390 10.1002/anie.201309258
- 391 34. Petkova, T., Hatanaka, M., Jaroniec, C. P., Hu, J. G., Belenky, M., Verhoeven, M., *et al.*
392 Tryptophan interactions in bacteriorhodopsin: a heteronuclear solid-state NMR study.
393 *Biochemistry* **41**, 2429-2437 (2002). DOI: 10.1021/bi012127m
- 394 35. Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P., & Lanyi, J. K. Structure of
395 bacteriorhodopsin at 1.55 Å resolution. *J. Mol. Biol.* **291**, 899-911 (1999). DOI:
396 10.1006/jmbi.1999.3027
- 397 36. Hashimoto, S., Obata, K., Takeuchi, H., Needleman, R., & Lanyi, J. K. Ultraviolet
398 resonance Raman spectra of Trp-182 and Trp-189 in bacteriorhodopsin: novel

- 399 information on the structure of Trp-182 and its steric interaction with retinal.
400 *Biochemistry* **36**, 11583-11590 (1997). DOI: 10.1021/bi971404f
- 401 37. Weidlich, O., Schalt, B., Friedman, N., Sheves, M., Lanyi, J. K., Brown, L. S., *et al.* Steric
402 interaction between the 9-methyl group of the retinal and tryptophan 182 controls 13-cis
403 to all-*trans* reisomerization and proton uptake in the bacteriorhodopsin photocycle.
404 *Biochemistry* **35**, 10807-10814 (1996). DOI: 10.1021/bi960780h
- 405 38. Kawamura, I., Kihara, N., Ohmine, M., Nishimura, K., Tuzi, S., Saito, H., *et al.* Solid-
406 state NMR studies of two backbone conformations at Tyr185 as a function of retinal
407 configurations in the dark, light, and pressure adapted bacteriorhodopsins. *J. Am. Chem.*
408 *Soc.* **129**, 1016-1017 (2007). DOI: 10.1021/ja0664887
- 409 39. Zhu, J., Lau, J. Y. C., & Wu, G. A solid-state ¹⁷O NMR study of L-tyrosine in different
410 ionization states: implications for probing tyrosine side chains in proteins. *J. Phys. Chem.*
411 *B* **114**, 11681-11688 (2010). DOI: 10.1021/jp1055123
- 412 40. Shigeta, A., Ito, S., Inoue, K., Okitsu, T., Wada, A., Kandori, H., *et al.* Solid-state nuclear
413 magnetic resonance structural study of the retinal-binding pocket in sodium ion pump
414 rhodopsin. *Biochemistry* **56**, 543-550 (2017). DOI: 10.1021/acs.biochem.6b00999
- 415 41. Ding X., Wang, H., Peng, B., Cui, H., Gao, Y., Iuga, D., *et al.* Mediation mechanism of
416 tyrosine 185 on the retinal isomerization equilibrium and the proton release channel in

- the seven-transmembrane receptor bacteriorhodopsin. *Biochim. Biophys. Acta, Bioenerg.* **1857**, 1786-1795 (2016). DOI: 10.1016/j.bbabbio.2016.08.002
42. Nishikawa, R., Kawamura, I., Okitsu, T., Wada, A., Sudo, Y., Kamo, N., et al. Functional conformation of Tyr residues in pharaonis phoborhodopsin as studied by solid state NMR. 16th International Conference on Retinal Proteins, Shiga, Japan, October 5-10, 2014, P-48.
43. Sudo, Y., Furutani, Y., Shimono, K., Kamo, N., & Kandori, H. Hydrogen bonding alteration of Thr-204 in the complex between pharaonis phoborhodopsin and its transducer protein. *Biochemistry* **42**, 14166-14172 (2003). DOI: 10.1021/bi035678g
44. Jang, D., El-Sayed, M. A., Stern, L. J., Mogi, T., & Khorana, H. G. Effect of genetic modification of tyrosine-185 on the proton pump and the blue-to-purple transition in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* **87**, 4103-4107 (1990). DOI: 10.1073/pnas.87.11.4103
45. Shimono, K., Ikeura, Y., Sudo, Y., Iwamoto, M., & Kamo, N. Environment around the chromophore in pharaonis phoborhodopsin: mutation analysis of the retinal binding site. *Biochim. Biophys. Acta -Biomembranes-* **1515**, 92-100 (2001). DOI: 10.1016/s0005-2736(01)00394-7
46. Hu, J. G., Sun, B. Q., Petokova, A. T., Griffin, R. G., & Herzfeld, J. The predischage

- 435 chromophore in bacteriorhodopsin: a ^{15}N solid-state NMR study of the L
436 photointermediate. *Biochemistry* **36**, 9316-9322 (1997). DOI: 10.1021/bi970416y
- 437 47. Eilers, M., Reeves, P. J., Ying, W., Khorana, H. G., & Smith, S. O. Magic angle spinning
438 NMR of the protonated retinylidene Schiff base nitrogen in rhodopsin: expression of ^{15}N -
439 lysine- and ^{13}C -glycine-labeled opsin in a stable cell line. *Proc. Natl. Acad. Sci. U. S. A.*
440 **96**, 487-492 (1999). DOI: 10.1073/pnas.96.2.487
- 441 48. Kawamura, I., Degawa, Y., Yamaguchi, S., Nishimura, K., Tuzi, S., Saitô, H., *et al.*
442 Pressure-induced isomerization of retinal on bacteriorhodopsin as disclosed by fast magic
443 angle spinning NMR. *Photochem. Photobiol.* **83** 346-350 (2007). DOI: 10.1562/2006-
444 06-20-rc-941
- 445 49. Shi, L., Kawamura, I., Jung, K.H., Brown, L.S., & Ladizhansky, V. Conformation of a
446 seven-helical transmembrane photosensor in the lipid environment. *Angew. Chem. Int.*
447 *Ed.* **50**, 1302-1305 (2011). DOI: 10.1002/anie.201004422
- 448 50. Shionoya, T., Mizuno, M., Tsukamoto, T., Ikeda, K., Seki, H., Kojima, K., *et al.* High
449 thermal stability of oligomeric assemblies of thermophilic rhodopsin in a lipid
450 environment. *J. Phys. Chem. B* **122**, 6945-6953 (2018). DOI: 10.1021/acs.jpcc.8b04894
- 451 51. Tomonaga, Y., Hidaka, T., Kawamura, I., Nishio, T., Osawa, K., Okitsu, T., *et al.* An
452 active photoreceptor intermediate revealed by in situ photoirradiated solid-state NMR

spectroscopy. *Biophys. J.* **101**, L50-L52 (2011). DOI: 10.1016/j.bpj.2011.10.022

52. Hayashi, S., Tajkhorshid, E., Pebay-Peyroula, E., Royant, A., Landau, E. M., Navarro, J.,

et al. Structural Determinants of Spectral Tuning in Retinal Proteins-Bacteriorhodopsin

vs Sensory Rhodopsin II. *J. Phys. Chem. B* **105**, 10124-10131 (2001). DOI:

10.1021/jp011362b

53. Shimono, K., Ikeura, Y., Sudo, Y., Iwamoto, M., & Kamo, N. Importance of the location

of the negative-charged counter-ion against the protonated Schiff base on the

chromophore configuration of pharaonis phoborhodopsin. *J. Photosci.* **9**, 302-304 (2002).

DOI: 10.1016/s0006-3495(02)75236-3

471

472 Figure legends

473 **Figure 1** Structure of all-trans retinal and surrounding residues in the retinal-binding site
474 of BR [PDB: 1C3W] (<https://doi.org/10.2210/pdb1c3w/pdb>) together with the
475 corresponding residues of MR (Ile47, Asp84, Trp182, Tyr185, Asp213, and Lys217). The
476 14-C, 20-C, and PSB positions are shown with dotted lines.

477

478 **Figure 2** (a) ^{13}C CP-MAS NMR spectra of stable-isotope labeled (black) and non-labeled
479 (red) MR in PG liposome at 10°C. SSB, spinning side band. (b) Expanded 14- ^{13}C
480 resonance in the 111-133 ppm region. (c) Expanded 20- ^{13}C resonance in the 3-32 ppm
481 region.

482

483 **Figure 3** Cross peaks observed in the ^{13}C - ^{13}C DARR NMR spectra of stable-isotope
484 labeled MR in the PG liposome over a mixing time of 500 ms. The sets of cross peaks for
485 20-C of all-*trans* and 13-*cis*, 15-*syn* isomers are represented using green and blue lines,
486 respectively.

487

488

489

490 **Figure 4** (a) ^{15}N CP-MAS NMR spectra of the dark-adapted MR in PG liposomes at pH
 491 7.0. (b) Expanded ^{15}N NMR signal of PSB in MR between 160 and 210 ppm is indicated
 492 in the upper panel. At the bottom, the PSB signals are shown in the upper interpreted via
 493 deconvolution with the two components that provided a good fit line (red line), i.e., all-
 494 *trans* (green line) and 13-*cis*, 15-*syn* (blue line). The chemical shifts of all-*trans* and 13-
 495 *cis*, 15-*syn* isomers are indicated using dotted lines.

496

497 **Figure 5** The relationships of ^{15}N chemical shifts of PSB in: (a) The all-*trans* isomer and
 498 (b) 13-*cis* isomer with the λ_{max} . The linear relationships were as follows: for all-*trans*
 499 model, (λ_{max} (nm) = -5.0872δ (ppm) + 1415.1); 13-*cis* model, (λ_{max} (nm) = -4.6709δ
 500 (ppm) + 1341.3) [46]. ^{15}N PSB chemical shift values of BR [48], KR2 [40], TR [50], and
 501 *Np*SRII [51] are recalibrated to the reference signal of $^{15}\text{NH}_4\text{Cl}$.

Figure 1 Structure of all-*trans* retinal and surrounding residues in the retinal-binding site of BR [PDB: 1C3W] (<https://doi.org/10.2210/pdb1c3w/pdb>) together with the corresponding residues of MR (Ile47, Asp84, Trp182, Tyr185, Asp213, and Lys217). The 14-C, 20-C, and PSB positions are shown with dotted lines.

296x209mm (300 x 300 DPI)

Figure 2 (a) ^{13}C CP-MAS NMR spectra of stable-isotope labeled (black) and non-labeled (red) MR in PG liposome at 10 °C. SSB, spinning side band. (b) Expanded $^{14}\text{-}^{13}\text{C}$ resonance in the 111-133 ppm region. (c) Expanded $20\text{-}^{13}\text{C}$ resonance in the 3-32 ppm region.

736x839mm (72 x 72 DPI)

Figure 3 Cross peaks observed in the ^{13}C - ^{13}C DARR NMR spectra of stable-isotope labeled MR in the PG liposome over a mixing time of 500 ms. The sets of cross peaks for 20-C of all-*trans* and 13-*cis*, 15-*syn* isomers are represented using green and blue lines, respectively.

1056x733mm (72 x 72 DPI)

Figure 4 (a) ^{15}N CP-MAS NMR spectra of the dark-adapted MR in PG liposomes at pH 7.0. (b) Expanded ^{15}N NMR signal of PSB in MR between 160 and 210 ppm is indicated in the upper panel. At the bottom, the PSB signals are shown in the upper interpreted via deconvolution with the two components that provided a good fit line (red line), i.e., all-*trans* (green line) and 13-*cis*, 15-*syn* (blue line). The chemical shifts of all-*trans* and 13-*cis*, 15-*syn* isomers are indicated using dotted lines.

794x823mm (72 x 72 DPI)

Figure 5 The relationships of ^{15}N chemical shifts of PSB in: (a) The all-*trans* isomer and (b) 13-*cis* isomer with the $\lambda_{\max}$. The linear relationships were as follows: for all-*trans* model; ($\lambda_{\max}$ (nm) = -5.0872δ (ppm) + 1415.1), 13-*cis* model ; ($\lambda_{\max}$ (nm) = -4.6709δ (ppm) + 1341.3) [46]. ^{15}N PSB chemical shift values of BR [48], KR2 [40], TR [50], and NpSRII [51] are recalibrated to the reference signal of $^{15}\text{NH}_4\text{Cl}$.

Graphical Abstract

229x131mm (300 x 300 DPI)