Article for *Photochemistry and Photobiology (Communication)*

Pressure induced isomerization of retinal on bacteriorhodopsin as disclosed by Fast Magic Angle Spinning NMR

Izuru Kawamura[1], Yoshiaki Degawa[1], Satoru Yamaguchi[2], Katsuyuki Nishimura[1], Satoru Tuzi[2], Hazime Saitô[2, 3] and Akira Naito[1*]

[1]Graduate School of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan, [2]Department of Life Science, University of Hyogo, Harima Science Garden City, Kamigori, Hyogo 678-1297, Japan, [3]Center for Quantum Life Sciences, Hiroshima University, Higashi-Hiroshima, Japan.

*Corresponding author. naito@ynu.ac.jp (A. Naito)

ABSTRACT

Bacteriorhodopsin (bR), a retinal protein in purple membrane of *H.salinarum*, shows functions as a light-driven proton pump. We have detected pressure induced isomerization of retinal in bR by ^{15}N cross polarization-magic angle spinning (CP-MAS) NMR spectra of [ε-^{15}N]Lys-labeled bR. In the ^{15}N NMR spectra, both all-*trans* and 13-*cis* retinal configurations have been observed at 148.0 and 155.0 ppm, respectively, at the MAS frequency of 4 kHz in the dark. When the MAS frequency was increased up to 12 kHz corresponding to the sample pressure of 80 atm, the ^{15}N NMR signals of Schiff Base of retinal were broadened. The signal intensity of 13-*cis* retinal at 155.0 ppm was increased when the MAS frequency was decreased from 12 kHz to 4 kHz. These results showed that the equilibrium constant of [13-*cis* –bR] /[all-*trans*-bR] increased by the pressure of 80 atm. It was also revealed that the changes induced by the pressure were quite local. Therefore, microscopically, hydrogen-bond network around retinal would be disrupted or distorted by a constantly applied pressure. It is, therefore, clearly demonstrated that increased pressure induced by fast MAS frequencies generated 13-*cis* isomerization of retinal in the membrane protein bR.

INTRODUCTION

Bacteriorhodopsin (bR), in *Halobacterium salinarum*, is a 26 kDa seven transmembrane helix protein with a retinal as a chromophore via Lys 216 in helix G and utilizes light to release proton by retinal photoisomerization from all-*trans* to 13-*cis*, 15-*anti* state [**1**]. The retinal configurations in the dark-adapted bR coexist as all-*trans* and 13-*cis*, 15-*syn* state with the isomeric ratio close to 1 [**2-4**]. Primary amino acid residues in the vicinity of Schiff base region of retinal are Arg82, Asp85, Tyr185, and Asp212[**5**] forming hydrogen-bond network with strongly hydrogen-bonded water molecules. BR assembles into naturally occurring 2D crystalline patches known as purple membrane (PM) in which its trimeric unit is hexagonally packed under the physiological condition [**6**]. 3D structure of bR with 1.55 Å resolutions has been revealed by X-ray diffraction [**7**]. To gain insight into the mechanism of proton pump activity, it is important to understand how retinal configurations influence conformation and dynamics of retinal protein in a molecular level.

The above-mentioned isomeric ratio of retinal in the dark-adapted state was shown to decrease from unity with the molar volume changes decrease by increased pressure [**8,9**]. Namely, pressure effects for bR in the dark showed that the isomer equilibrium shifts from all-*trans* towards 13-*cis* state at two processes with the molar volume changes of -7 and -21 mL/mol [**10**]. It is expected that the molar volume change of the first step is quite large, so that even in the small pressure change would give a large change of the population between 13-*cis* and all-*trans* state. The isomeric ratio in the dark, however, is insensitive to the temperature change [**11, 12**] and bR is thermally very stable [**13**], although high temperature intermediate in bR exists at a temperature > 60℃ [**14**].

We have revealed that local conformation and dynamics by ^{13}C NMR study of amino-acid selective isotope-labeled bR [**15-17**]. In particular, it turned out that ^{13}C NMR peaks were well-resolved for fully hydrated [3-^{13}C]Ala-, [1-^{13}C]Val-labeled bR, depending upon their local dynamics and conformations of 29 Ala or 21 Val residues, respectively [**18,19**]. In this study, pressure effects as isomeric ratio of retinal for [ε-^{15}N]Lys-labeled bR in the dark were detected under fast magic angle spinning (MAS) condition. MAS can eliminate the chemical shift anisotropy when the rotation axis of sample is inclined at 54.7° to the static magnetic field and the rotor frequency is faster than the chemical shift anisotropy [**20,21**]. Pressure effects induced by the centrifugal forces in the MAS in solids have been reported in a few case. First, interlamellar waters in multilamellar vesicles (DOPC, POPC) with spinning frequency in ^{1}H MAS NMR decrease on dispersions [**22**]. Second, dynorphin (opioid peptide; 17 residues) has partially dissociated from lipid bilayers on ^{13}C MAS NMR condition. In the static condition, the peptide binds to membrane,and consequently forms magnetically oriented vesicle systems (MOVS) [**23,24**].

Here, we will focus our attention on pressure effects for bR in PM by solid state MAS NMR measurements. Retinal configurations in bR were revealed by ^{15}N NMR spectra of [ε-^{15}N]Lys-labeled bR under fast MAS conditions. We consider that this experimental system applies constantly low pressure for fully hydrated sample and the pressure of 80 atm was induced at the MAS speed of 12 kHz.

MATERIALS AND METHODS

Halobacterium salinarum S9 was grown in temporary synthetic medium including [ε-^{15}N]-L-Lys to yield [ε-^{15}N]Lys-labeled bR in purple membrane (PM). PM was isolated by the standard method as described [**25**] and suspended in 5 mM HEPES buffer containing 0.02 % NaN_3 and 10 mM NaCl at pH 7. The sample was concentrated by centrifugation and placed in 4.0 mm o.d. zirconia pencil-type rotor for fast magic angle spinning (MAS) experiments. Sample rotor was tightly shielded by optical-fiber cap and glued to the rotor by rapid Alardyte® to prevent dehydration of pelleted samples and *in situ* light-illumination to sample was made through optical fiber.

^{15}N high-resolution solid-state NMR spectra were recorded on a Chemagnetics CMX-400 infinity FT-NMR spectrometer operating at 100.16 MHz for carbon and 40.3 MHz for nitrogen nuclei, by cross polarization-magic angle spinning (CP-MAS) on variable amplitude contact pulse of observed nucleus [**26**]. A double resonance MAS probe equipped with 4.0 mm o.d. rotor was used for all measurements. The spinning frequencies were set to 4-12 kHz and 90 ° pulse was 5.1 μs. The MAS NMR measurements were performed at 4 kHz after the measurements at 12 kHz. Ambient conditions of MAS were set at 4 kHz and probe setting temperature at 20℃. Variable amplitude CP-MAS with two pulse phase modulation (TPPM) decoupling [**27**] was employed. ^{15}N contact time and repetition time were 2 ms, and 4 s, respectively. ^{15}N chemical shifts were externally referred to 11.59 ppm for the amino nitrogen of glycine from NH_4NO_3.

Pressure on the samples was naturally applied by the centrifugal force induced by MAS frequency. Since the centrifugal force is proportional to the diameter of rotor and the square of rotor frequency, pressure of inner wall of the rotor with 4mm o.d. can be

estimated to be 9 atm and 80 atm for the rotor frequency of 4 and 12 kHz, respectively.

RESULTS AND DISCUSSION

Figure 1 (A)-(D) shows ^{15}N CP-MAS NMR spectra of [ε-^{15}N]Lys-labeled bR at the spinning frequencies of 4, 8, 10 and 12 kHz in the dark. After spinning sample at the MAS frequency of 12 kHz, CP-MAS spectrum was recorded at 4 kHz to examine pressure effects on retinal configurations (Figure 1 (E)). The ^{15}N signals of free lysine and backbone amides of naturally abundant amino acid residues were observed at 11.0 and 90-110 ppm, respectively. The signal intensities of the free lysine largely decreased at the MAS frequencies of 10 and 12 kHz as compared with those of backbone components, in which the Hartmann-Hahn matching condition $H_{1H}\gamma_H=H_{1N}\gamma_N$ were disturbed by the fast MAS. When the MAS frequency was returned to 4 kHz, the intensities and chemical shifts of these signals were recovered and they did not show any hysteresis.

Figure 2 shows the ^{15}N CP-MAS spectra of [ε-^{15}N]Lys-bR in the spectral range between 100 and 200 ppm. The signals of the protonated Schiff base are shown in the Figure as a sensitive probe on the changes of retinal isomerization and environment (Figure 2(A)-(D)) [**10, 28, 29**]. Both ^{15}N NMR signals due to all-*trans* and 13-*cis* retinal configurations were observed at 148.0 and 155.0 ppm under the MAS frequency of 4 kHz (Figure 2(A)). When the MAS frequency was increased up to 12 kHz, the ^{15}N NMR signals of Schiff base of retinal showed broadening (Figure 2(B)). This finding indicates that the hydrogen-bond network around the protonated Schiff base is locally distorted with the induced pressure by the fast MAS (Figure 3). The signal intensity of the 13-*cis* retinal at 155.0 ppm increased as the MAS frequency was decreased from 12 kHz to 4 kHz (Figure 1 (E) and 2 (C)). Retinal configurations caused hysteresis by the

fast MAS experiments. To assign the ^{15}N NMR signals to either 13-*cis* or all-*trans* configuration, a white light was irradiated continuously to bR and significant increase of the signal of all-*trans* was observed (Figure 2(D)). It was shown that the all-*trans* retinal configuration could be confirmed by examination of the ^{15}N peak-position by illumination of white light. Therefore, it turned out that retinal configurations of bR changed from the all-*trans* to 13-*cis* retinal of [ε-^{15}N]Lys-labeled bR by fast MAS experiments. Obviously, the present observation suggests that the pressure induced isomerization of retinal causes distortion of such hydrogen-bond networks in the vicinity of retinal (Figure 3) [7], together with a possible dehydration effect of surrounding water molecules by centrifuging effect of fast MAS. It was clearly demonstrated that the proportions of retinal isomers are almost the same as the dark-adapted state bR, which is shifted from all-*trans* to 13-*cis* state by the application of pressure [**8,9**].

Isomerization from all-*trans* to 13-*cis* has been studied by extraction techniques and absorption spectroscopy of the range from low to high pressure (100-3000 atm) in which the isomer equilibrium shifts from all-*trans* to 13-*cis* state at two processes with the molar volume changes in the dark [**10**]. Thermodynamically, this change of equilibrium constant of [13-*cis*]/[all-*trans*] can be attributed to the large molar volume change from all-*trans* to 13-*cis* state. Microscopically, locations of water molecules in the vicinity of retinal [**7**] and hydrogen-bond networks via water molecules that correlate with proton pump activity may be distorted by the applied pressure. In solution high-pressure ^{15}N/^{1}H NMR techniques utilizing pressure cell, fluctuations of hen lysozyme are found to be localized near the cavities containing water molecules [**30,31**]. In general, hydrogen bonds of protein structure changed H···O or H···N distances to

pressure with molar volume change [**31**]. Therefore, the pressure induced isomerization of retinal and modulation of the hydrogen-bond network can cause the change in motion of residues located in the vicinity of retinal.

Noticeable pressure effects on bR in purple membrane in the molecular level were observed at the first time in this experiments. Increased pressure by the fast MAS frequencies induces isomerization from the all-*trans* to 13-*cis* retinal. It is possible to increase 13-*cis* state by applying pressure and the all-*trans* state by irradiation of light. It is stressed that the MAS experiment is a good source of pressure since the induced pressure will be proportional to the square of MAS frequency. This kind of pressure effect in membrane protein has not yet been studied because it was thought that the application of pressure in the solid state NMR spectroscopy was difficult. This experiment, therefore, clearly showed that pressure induced experiment can be successfully applied by the fast MAS experiment. It is also important to point out that it is possible to change the equilibrium state by applying pressure because a large molar volume change in a large heterogeneous system such as membrane protein can be change the equiliburium state by relatively small change of pressure.

ACKNOWLEDGEMENTS

The present work was supported by a Grant-in-Aid for Scientific Research on Priority Area (No. 17048010) from MEXT, Japan and for Scientific Research (No. 17370054) from JSPS, and by Research Grant-in-Aid from Magnetic Health Science Foundation. I. K. thanks young grant for “Research Aid Program for Ph. D. Candidates” on Yokohama National University.

References

1. Lanyi, J. K.and B. Schobert (2004) Local-global conformational coupling in a heptahelical membrane protein: Transport mechanism from crystal structure of the nine states in the bacteriorhodopsin photocycle, *Biochemistry* **43** 3-8.

2. Maeda, A., T. Iwasa, and T. Yoshizawa (1977) Isomeric composition of retinal chromophore in dark-adapted bacteriorhodopsin, *J. Biochem.* **82** 1599-1604.

3. Trissl, H-W. and W. Gartner (1987) Rapid charge separation and batochromic absorption shift of flash-excited bacteriorhodopsin containing 13-*cis* or all-*trans* forms of substituted retinals, *Biochemistry* **26** 751-758.

4. De Groot, H. J. M., G. S. Harbison, J. Herzfeld, and R. G. Griffin (1989) Nuclear magnetic resonance study of the Schiff base in bacteriorhodopsin: Counterion effects on the ^{15}N shift anisotropy, *Biochemistry* **28** 3346-3353.

5. Shibata M., and H. Kandori (2005) FTIR studies of internal water molecules in the Schiff base region of bacteriorhodopsin, *Biochemistry* **44** 7406-7413.

6. Grigorieff, N., T. A. Ceska, K. H. Downing, J. M. Baldwin, R. Henderson (1996) Electron-crystallographic refinement of the structure of bacteriorhodopsin, *J. Mol. Biol.* **259** 393-421.

7. Luecke, H., B. Schobert, H-T. Richter, J-P. Cartailler and J. K. Lanyi (1999) Structure of bacteriorhodopsin at 1.55 Å resolution, *J. Mol. Biol.* **291** 899-911.

8. Tsuda, M., and T. G. Ebrey (1980) Effect of high pressure on the absorption spectrum and isomeric composition of bacteriorhodopsin, *Biophys. J.* **30** 149-158.

9. Tsuda, M., R. Govindjee, T. G. Ebrey (1983) Effects of pressure and temperature on the M412 intermediate of the bacteriorhodopsin photocycle. Implications for the phase transition of the purple membrane, *Biophys. J.* **44** 249-254.

10. Bryl, K., and K. Yoshihara (2002) Two processes lead to a stable all-*trans* and 13-*cis* isomer equilibrium in dark-adapted bacteriorhodopsin; effect of high pressure on bacteriorhodopsin, bacteriorhodopsin mutant D96N and fluoro-bacteriorhodopsin analogues, *Eur. Biophys. J.* **31** 539-548.

11. Dencher, N. A., Ch. N. Rafferty and W. Sperling (1977) 13-cis and trans bacteriorhodopsin: photochemistry and dark equilibrium, *Biophys. Struct. Mechan.* **3** 79-94

12. Ohno, K., Y. Takeuchi, M. Yoshida (1977) Light-induced formation of the 410 nm intermediate from reconstitued bacteriorhodopsin, *J. Biochem. (Tokyo)* **82** 1177-1180.

13. Mukai, Y., N. Kamo, and S. Mitaku (1999) Light-induced denaturation of bacteriorhodopsin solubilized by octyl-β-glucoside, *Protein Eng.* **12** 755-759.

14. Yokoyama, Y., M. Sonoyama, and S. Mitaku (2002) Irreversible photobleaching of bacteriorhodopsin in a high-temperature intermediate state, *J. Biochem.* **131** 785-90.

15. Saitô, H., S. Tuzi, S. Yamaguchi, M. Tanio and A. Naito (2000) Conformation and backbone dynamics of bacteriorhodopsin revealed by ^{13}C-NMR, *Biochem. Biophys. Acta.* **1460** 39-48.

16. Saitô, H., J. Mikami, S. Yamaguchi, M. Tanio, A. Kira, T. Arakawa, K. Yamamoto, and S. Tuzi (2004) Site-directed ^{13}C solid-state NMR studies on membrane proteins: strategy and goals toward revealing conformation and dynamics as illustrated for bacteriorhodopsin labeled with [1-^{13}C]amino acid residues, *Magn. Reson. Chem.* **42** 218-230.

17. Saitô, H., S. Yamaguchi, H. Okuda, A. Shiraishi and S. Tuzi (2004) Dynamic aspect of bacteriorhodopsin as a typical membrane protein as revealed by site-directed

solid-state ^{13}C NMR, *Solid State Nucl. Magn. Reson.* **25** 5-14.

18. Saitô, H., S. Tuzi and A. Naito (1998) Empirical versus nonempirical evaluation of secondary structure of fibrous and membrane proteins by solid-state NMR: a practical approach, *Annu. Rep. NMR Spectrosco.* **36** 79-121.

19. Saitô, H., S. Tuzi, M. Tanio, A. Naito (2002) Dynamic aspects of membrane proteins and membrane-associated peptides as revealed by ^{13}C NMR: Lessons from bacteriorhodopsin as an *intact* protein, *Annu. Rep. NMR Spectrosco.* **47** 39-108.

20. Andrew, E. R., Bradbury, A. and Eades, R. G. (1958) Nuclear magnetic resonance spectra from a crystal rotated at high speed, *Nature* **182** 1659.

21. Lowe, I. J. (1959) Free induction decays of rotating solids, *Phys. Rev. Lett.* **2** 285-287.

22. Zhou, Z., Sayer, R. G., Hughes, D.W., Stark, R. E. and Epand, R.M (1999) Studies of phospholipid hydration by high-resolution magic-angle spinning nuclear magnetic resonance, *Biophys. J.* **76** 387-399.

23. Naito, A., T. Nagao, M. Obata, Y. Shindo, M. Okamoto, S. Yokoyama, S. Tuzi, and H. Saitô (2002) Dynorphin induced magnetic ordering in lipid bilayers as studied by ^{31}P NMR spectroscopy, *Biochim. Biophys. Acta.* **1558** 34-44.

24. Uezono, T., S. Toraya, M. Obata, K. Nishimura, S. Tuzi, H. Saitô and A. Naito (2005) Structure and orientation of dynorphin bound to lipid bilayers by ^{13}C solid-state NMR., *J. Mol. Struct.* **749** 13-19.

25. Oesterhelt D., and W. Stoeckenius (1974), Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane, *Methods Enzymol.* **31** 667-678.

26. Metz, G., X. Wu and S. O. Smith (1994) Ramped amplitude cross polarization in

magic angle spinning NMR, *J. Magn. Reson. ser. A* **110** 219-227.

27. Bennet, A. W., Rienstra, C. M., Augar, M., Lakshmi, K. V., and Griffin, R. G. (1995) Heteronuclear decoupling in rotating solids, *J. Chem. Phys.* **103** 6951-6958.

28. Smith, S. O., H. J. M. De Groot, R. Gebhard, J. M. L. Courtin, I. Lugtenburg, J. Herzfeld and R. G. Griffin (1989) Structure and protein environment of the retinal chromophore in light- and dark-adapted bR studied by solid state NMR, *Biochemistry* **28** 8897-8904.

29. Hatcher, M. E., J. G. Hu, M. Belenky, P. Verdegem, J. Lugtenburg, R. G. Griffin, and J. Herzfeld (2002) Control of the pump cycle in bacteriorhodopsin: Mechanisms elucidated by solid-state NMR of the D85N mutant, *Biophys. J.* **82** 1017–1029.

30. Kamatari Y. O., Yamada, H., Akasaka, K., Jones, J. A., Dobson, C. M. and Smith, L. J. (2001) Response of native and denatured hen lysozyme to high pressure studied by $^{15}N/^{1}H$ NMR spectroscopy, *Eur. J. Biochem.* 268:1782-1793.

31. Akasaka, K. (2003) Highly fluctuating protein structures revealed by variable-pressure nuclear magnetic resonance, *Biochemistry* 42:10875-10885.

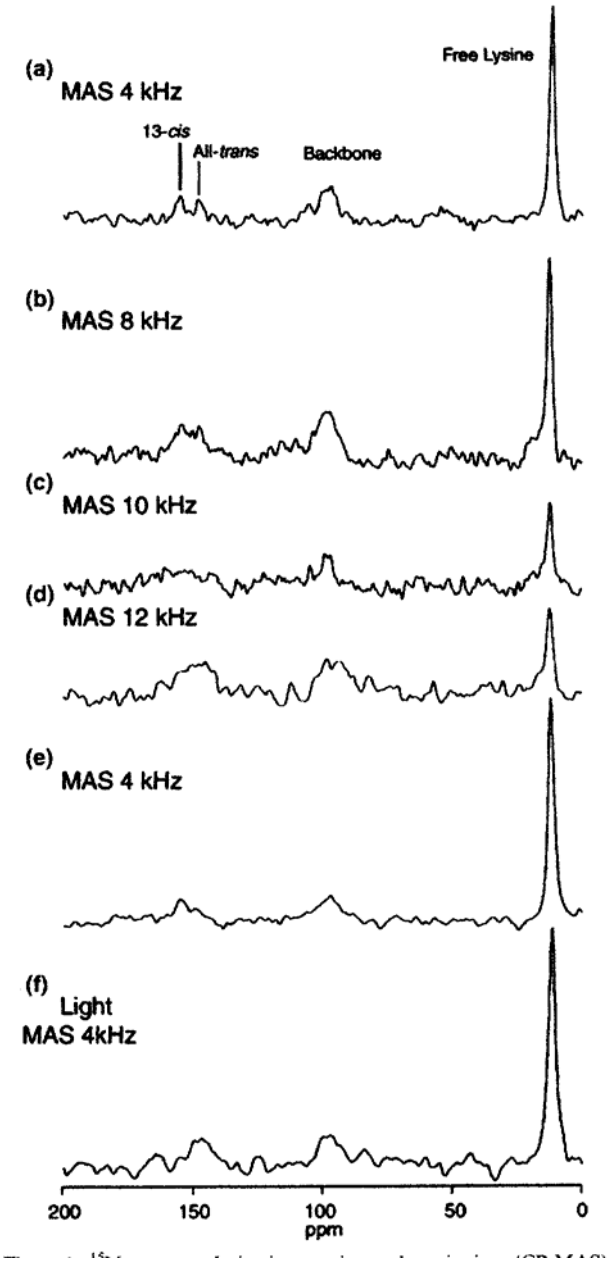

Figure 1. ^{15}N CP-MAS NMR spectra of [ε-^{15}N]Lys-labeled bR at various magic angle spinning frequencies up to 12 kHz ((A)-(D)). After spinning sample at 12 kHz, ^{15}N CP-MAS NMR spectrum was recorded at 4 kHz (E). *In situ* light-irradiation measurement of bR (F).

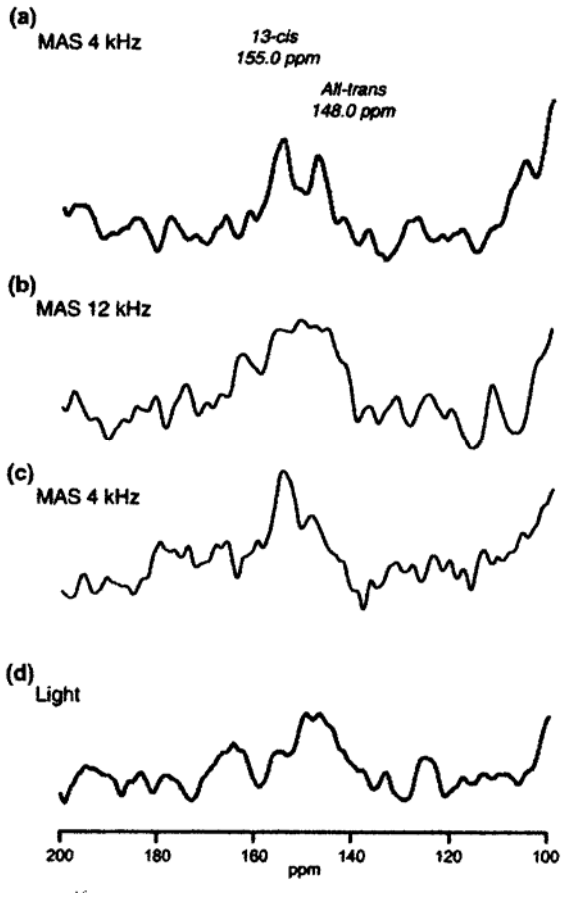

Figure 2 ^{15}N CP-MAS NMR spectra of [ε-^{15}N]Lys-labeled bR at MAS frequency of 4 and 12 kHz, respectively ((A) and (B)). After spinning sample at 12 kHz, CP-MAS NMR spectrum was observed at 4 kHz (C). *In situ* light-irradiation measurement of bR (D).

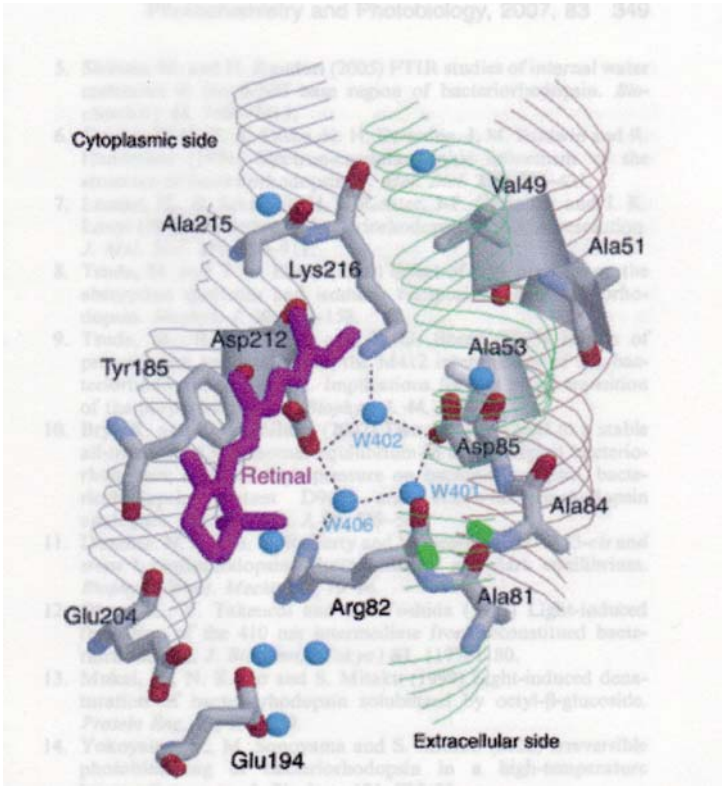

Figure 3. Schematic representation of primary amino acid residues and water in the vicinity of retinal.