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Solid-State NMR Studies of Two Backbone Conformations at Tyr185 as a Function of Retinal Configurations in the Dark, Light, and Pressure Adapted Bacteriorhodopsins

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Bacteriorhodopsin (bR), from *Halobacterium salinarum*, is a 26 kDa membrane protein that consists of seven transmembrane helices with a retinal covalently linked to Lys216 through protonated Schiff base. The bR absorbs light to cause retinal photoisomerization from the all-*trans* to 13-*cis*, 15-*anti* state followed by sequential proton-transfer reactions.[1] The retinal configuration in the dark-adapted bR coexists as all-*trans* and 13-*cis*, 15-*syn* states with the isomeric ratio close to 1.[2] When illuminated (560 nm), the population of the all-*trans* state is increased. This all-*trans* populated state is called a light adapted state. The population of the 13-*cis*, 15-*syn* state can be increased when pressure is increased.[3,4] Here, we refer to the 13-*cis* populated state as a pressure adapted state. X-ray diffraction study shows that primary amino acid residues Arg82, Asp85, Tyr185, and Asp212 and water molecules W401, W402, and W406 are located in the vicinity of the Schiff base region of retinal.[5] It has been revealed that helix F, including Pro186 in the hinge region, moves outward by a tilt motion to open the proton channel at the cytoplasmic side in the M intermediate. Tyr residues in bR are known to be important for the photocycle and regulate color tuning; in particular, Tyr57, 83, and 185 are located in the vicinity of retinal.[6] When these Tyr residues are replaced by other residues, the proton release rate is markedly delayed.[7] This clearly indicates that tyrosine plays an important role in proton pump activity.

Rotational echo double resonance (REDOR) filtered experiments[8] in solid-state NMR are a powerful method to observe a NMR peak of a specific nuclear pair with strong dipolar interaction by using doubly isotopic labeling of a unique consecutive amino acid sequence in a protein. Namely, the NMR peak obtained from the difference spectrum between REDOR and full echo spectra show the signal of a labeled nucleus in a particular amino acid that is directly forming a peptide bond to the other labeled nucleus.[8] Moreover, conformation dependent ^{13}C isotropic chemical shifts of [1-^{13}C]Tyr have been used to determine secondary structure in the vicinity of Tyr residues.[9] In this paper, we use a REDOR filter to allow selective observation of Tyr-X peptide bonds in bR and characterize the backbone conformation of bR with respect to retinal-protein interactions. We also observed the Tyr signals corresponding to the dark, light, and pressure adapted states of bR. Here, in situ continuous photoillumination was made by an optical fiber from outside the magnet through a tightly sealed piece of cap made of optical fiber glued to the rotor. Pressure on the samples was naturally applied by the centrifugal force induced by the MAS frequency. Since the centrifugal force is proportional to the square of rotor radius and the square of rotor frequency, the pressure at the inner wall of this rotor with 5 mm o.d. can be estimated to be 12 bar for the rotor frequency of 4 kHz.

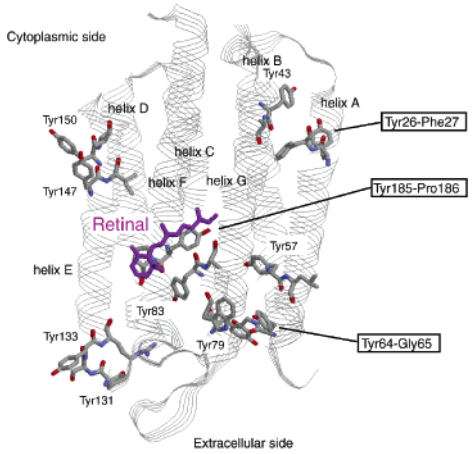

Figure 1. There are 11 Tyr residues in bR and Tyr-X peptide bond is unique in the sequence except when X is Ala. The three pairs of Tyr-X peptide bonds in bR for the REDOR filter experiments: Tyr26-Phe27, Tyr64-Gly65, and Tyr185-Pro186. Tyr185 lies close to retinal. (PDB code: 1C3W[6]).

The doubly isotopically labeled samples, (a) [1-^{13}C]Tyr/[^{15}N]-Phe-bR, (b) [1-^{13}C]Tyr/[^{15}N]Gly-bR, and (c) [1-^{13}C]Tyr/[^{15}N]Pro-bR, were prepared from *H. sarinarum* in the TS medium with [1-^{13}C]Tyr and [^{15}N]X, where X is Phe, Gly, or Pro. Tyr185-Pro186 is located in the vicinity of retinal and a helix kink point in the photocycle. Tyr64-Gly65 is located at the membrane surface on the extracellular side and Tyr26-Phe27 in helix A at the cytoplasmic side (Figure 1). These samples were prepared in 5 mM HEPES, 10 mM NaCl (pH7.0) as excess humidity membrane pellets.[10] ^{13}C REDOR-filtered experiments were performed on a Chemagnetics CMX-400 Infinity FT-NMR spectrometer at the MAS spinning frequency of 4 kHz.

Figure 2 shows REDOR filtered ^{13}C NMR spectra of [1-^{13}C]-Tyr/[^{15}N]X-bR. It is interesting to note that the REDOR-filtered spectrum of [1-^{13}C]Tyr/[^{15}N]Pro-bR obviously showed two peaks at 173.4 and 177.7 ppm corresponding to the [1-^{13}C] Tyr185 with an intensity ratio of 1:1 (Figure 2C). This finding is attributed to the presence of two retinal configurations in the dark adapted state. In fact, Tyr185 closely lies in the vicinity of retinal.[2,6] By contrast, the spectra of Tyr26 (Figure 2A) and Tyr64 (Figure 2B) of bR showed a single peak at 174.0 and 175.2 ppm, respectively. Tyr26

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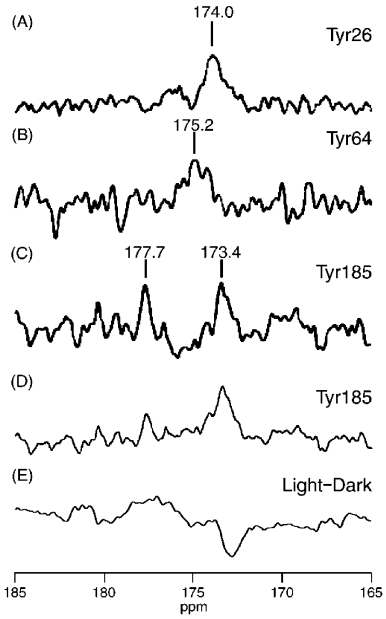

Figure 2. ^{13}C REDOR filtered spectra of (A) [1-^{13}C]Tyr/[^{15}N]Phe-bR, Tyr26; (B) [1-^{13}C]Tyr/[^{15}N]Gly-bR, Tyr64; (C) [1-^{13}C]Tyr/[^{15}N]Pro-bR, Tyr185; and (D) [1-^{13}C]Tyr/[^{15}N]Pro-bR with pressure following longer accumulation (60 h), Tyr185 (13-*cis* retinal rich). The peak obtained from difference spectra between full echo and REDOR experiments can distinguish a unique isotropic signal of directly bonded $^{13}C-^{15}N$ from others using short NcTr = 2 ms. Trace E is the ^{13}C CP-MAS difference NMR spectra between light and dark at ambient temperature. The ^{13}C chemical shifts are referenced to that of TMS as 0 ppm.

and Tyr64 are considered to be not sensitive to retinal configurations because they are located far from the retinal as shown in Figure 1. These spectra indicated that not only retinal configurations but also bR backbone conformations coexist in two states corresponding to the all-*trans* and 13-*cis*, 15-*syn* retinal configurations.

Notably, for the two peaks of Tyr185, the isotropic ^{13}CO chemical shift values are considerably different. The peak at 177.7 ppm is attributed to the all-*trans* retinal configuration, according to ^{13}C REDOR-filtered experiments in the light adapted state.[8] This unusually high chemical shift value of 177.7 ppm (176.7 ppm for α-helix in Tyr)[9] indicates α-helix structure with steric hindrance from all-*trans* retinal or with a hydrogen bond such as a bifurcated one.[9] On the other hand, the peak at 173.4 ppm is influenced by the 13-*cis* retinal configuration and the shift value indicates a non α-helical structure or a highly distorted α-helix.[9] The Schiff base region forms a pentagon cluster with strong hydrogen bond networks between Asp85, Asp212, and three water molecules.[5,6] Tyr185 forms a hydrogen bond with Asp212, but the Y185F mutant may not perturb the pentagon cluster as disclosed by FTIR difference absorbance data.[5] As a result of REDOR-filtered experiments, the backbone conformation near Tyr185 may change pronouncedly although the same pentagon hydrogen-bond networks are retained.

REDOR-filtered ^{13}C NMR resonance of Tyr26 appeared as a single peak at 174.0 ppm. This value may indicate a dynamic α-helical conformation with 13-*cis* retinal. An increase in CO···HN hydrogen-bond length causes an upfield shift of the isotropic ^{13}C chemical shift value. It is, therefore, considered that the Tyr26 CO···Lys30HN length is slightly elongated and the region near Tyr26 has formed a disordered structure or dynamic α-helix.[10,11] The chemical shifts of Tyr64 indicated α-helical structure, although Tyr64 is located in the region of the extracellular membrane surface.

When the REDOR difference spectrum under MAS condition was taken with a long accumulation time of 60 h, the signal at 173.4 ppm for Tyr185 increased markedly (Figure 2D). This observation can be attributed to the pressure induced isomerization of retinal from the all-*trans* to 13-*cis*, 15-*syn* state.[3] This pressure induced isomerization has been observed by analyzing the photoisomer of retinal by chromatography[3] and MAS NMR,[4] although pressure induced isomerization occurs at much lower pressure than the reported pressure in this experiment. It is noted that the protein rather than just retinal also changes conformation accompanied by the change of retinal configuration by pressure.

Figure 2E shows the difference spectrum between the dark and light adapted states using in situ photoillumination experiments. Photoillumination can change the retinal isomer from the 13-*cis* to all-*trans* configuration which subsequently induces the change of backbone conformation in bR. Line broadening was observed at 177.7 ppm, which can be attributed to the short lifetime of the all-*trans* state because this species can be transferred to the other excited states during photoillumination. It is also possible to cause broadening by the distribution of slightly different configurations of all-*trans* retinal and the overlapping of other Tyr residues.

In conclusion, backbone conformations of Tyr185 in bR were strongly perturbed by the retinal configurations as disclosed from REDOR-filtered experiments. Consequently, two different conformations of bR coexist near Tyr185 corresponding to the all-*trans* and 13-*cis* configuration of retinal. The populations of the two conformations of bR changes to the all-*trans* populated state for the light adapted state, while the 13-*cis* populated state is dominant for the pressure adapted state. This local change of protein conformation was disclosed to be generated by photoisomerization of the retinal by means of the photoillumination experiments. In contrast, only single conformations for Tyr26 and Tyr64 appeared. It is, therefore, revealed that the change of retinal configuration may induce a significant local change of backbone conformation in bR that is relevant to the regulation of a light driven proton pathway.

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