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Dynamic aspects of extracellular loop region as a proton release pathway of bacteriorhodopsin studied by relaxation time measurements by solid state NMR

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Abstract

Local dynamics of interhelical loops in bacteriorhodopsin (bR), the extracellular BC, DE and FG, and cytoplasmic AB and CD loops, and helix B were determined on the basis of a variety of relaxation parameters for the resolved ^{13}C and ^{15}N signals of [1-^{13}C]Tyr-, [^{15}N]Pro- and [1-^{13}C]Val-, [^{15}N]Pro-labeled bR. Rotational echo double resonance (REDOR) filter experiments were used to assign [1-^{13}C]Val-, [^{15}N]Pro signals to the specific residues in bR. The previous assignments of [1-^{13}C]Val-labeled peaks, 172.9 or 171.1 ppm, to Val69 were revised: the assignment of peak, 172.1 ppm, to Val69 was made in view of the additional information of conformation-dependent ^{15}N chemical shifts of Pro bonded to Val in the presence of ^{13}C–^{15}N correlation, although no assignment of peak is feasible for ^{13}C nuclei not bonded to Pro. ^{13}C or ^{15}N spin–lattice relaxation times (T_1), spin–spin relaxation times under the condition of CP-MAS (T_2), and cross relaxation times (T_{CH} and T_{NH}) for ^{13}C and ^{15}N nuclei and carbon or nitrogen-resolved, ^1H spin–lattice relaxation times in the rotating flame (^1H $T_{1\rho}$) for the assigned signals were measured in [1-^{13}C]Val-, [^{15}N]Pro-bR. It turned out that V69–P70 in the BC loop in the extracellular side has a rigid β-sheet in spite of longer loop and possesses large amplitude motions as revealed from ^{13}C and ^{15}N conformation-dependent chemical shifts and T_1, T_2, ^1H $T_{1\rho}$ and cross relaxation times. In addition, breakage of the β-sheet structure in the BC loop was seen in bacterio-opsin (bO) in the absence of retinal.

Keywords: Bacteriorhodopsin; Bacterio-opsin; Relaxation time; Rotational echo double resonance; Solid state NMR

1. Introduction

Bacteriorhodopsin (bR) is a membrane protein from purple membrane (PM) of *Halobacterium salinarum* and consists of apo-protein, bacterio-opsin (bO), with seven transmembrane α-helices and retinal covalently linked to K216 of bR through a protonated Schiff base [1–3]. bR shows a function as a light-driven proton pump from the extracellular side to the cytoplasmic side. bR forms a specific component of PM in 2D arrays and its 3D structure was initially determined by cryoelectron microscope [4] and later by X-ray diffraction [5]. A proton is transported initially from the protonated Schiff base to D85. Simultaneously, protons are released from the proton release groups of E194 and E204 to the extracellular side. Hydrogen-bond networks are formed from the Schiff base region to the extracellular surface region through D85, R82, D212, Y185, Y57, E194, and E204, and water molecules play a crucial role in the proton transport of bR [3,5–7]. In addition, interhelical loops in bR also play an important role for its function and stability [8–10]. In particular, the BC loop of the extracellular side near the proton release group in bR forms a β-sheet [4,5] and is responsible for its function and thermal stability. To gain insight into the proton pump activity of bR, it is essential to understand the dynamics of extracellular loops to evaluate the interaction of apo-protein with retinal.

Abbreviations: bR, bacteriorhodopsin; bO, bacterio-opsin; PM, purple membrane; CP-MAS, Cross polarization-magic angle spinning; REDOR, Rotational echo double resonance

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Solid state NMR spectroscopy provides one valuable information on the conformation and dynamics of membrane proteins in lipid bilayers, because their crystallization for X-ray diffraction is not easy and the resulting molecular weight is too large for solution NMR [11–17]. In fact, we have analyzed the dynamic structure of bR with reference to our previous studies by site-directed ^{13}C solid state NMR. Fully hydrated bR in PM or reconstituted in lipid bilayer shows a variety of local motions in the order of 10^2–10^8 Hz, depending upon its particular sites, which may be related with the proton pump activity [18–20]. In particular, well-resolved ^{13}C NMR signals are available from [3-^{13}C]Ala-bR in PM, owing to their restricted motional frequencies in the transmembrane α-helices in the order of 10^2 Hz [21–23]. Further, it was revealed that substantial local dynamics of bR is caused by removal of retinal, site-directed mutagenesis, and a variety of environmental factors such as pH, temperature, metal ions, etc. [24–26]. We have subsequently observed that the cytoplasmic surface dynamics of [3-^{13}C]Ala residues are altered by site-directed mutations of A160G, E166G, A168G and R227Q, and a manner of cation binding. Thus, cytoplasmic surface regions of the C-terminal α-helix and cytoplasmic loops formed proton binding clusters and underwent concerted motions among the residues connecting with hydrogen bond networks [26].

REDOR (rotational echo double resonance) filter experiment is a powerful method to be able to locate NMR peaks from certain specific peptide bonds in membrane proteins by examination of a fast decay of the nuclear pair with a short distance [27–29]. During REDOR evolution time, the magnetization of a nuclear pair with short distance can decay very fast as compared with the other nuclear pairs with longer distances, caused by recoupling of heteronuclear dipolar interactions under MAS condition [30–34]. The difference spectra between the full echo and REDOR experiments can distinguish directly bonded ^{13}C–^{15}N pairs among those of ^{13}C–^{15}N pairs with long distances as far as a short REDOR evolution time is used. Recently, we have revealed by REDOR filter experiment that two different backbone conformations of bR coexist at Y185 corresponding to two retinal configurations such as all-*trans* and 13-*cis*, and 15-*syn* forms in the dark. We further found that the light adapted state contains all-*trans* form, while the pressure adapted state dominates 13-*cis*, and 15-*syn* forms [27,35].

We aimed to elucidate local dynamics of the extracellular loops of bR such as the BC and FG loops, because these regions play an important role in the proton release process. For this purpose, we have performed REDOR filter experiments on a ^{13}C–^{15}N nuclear pair of [1-^{13}C]Val-, [^{15}N]Pro-labeled bR to assign their NMR signals to consecutive Val-Pro sequences with the direct ^{13}C–^{15}N bond in bR. We further characterized the dynamic feature of such regions in view of various relaxation parameters for the shorter FG and longer BC loops and helix B. In addition, we further examined the ^{15}N and ^{13}C NMR spectra of [1-^{13}C]Tyr, [^{15}N]Pro-labeled bacterio-opsin (bO) to clarify a manner of interaction of retinal with the extracellular side.

2. Materials and methods

2.1. Materials

H. salinarum S9 was grown in temporary synthetic medium including [1-^{13}C]valine and [^{15}N]proline as sources of isotopically labeled, amino acids to yield selectively labeled [1-^{13}C]Val-, [^{15}N]Pro-labeled bR in PM. The doubly, isotopically labeled samples contain direct ^{13}C–^{15}N peptide bonds as three consecutive amino acid sequences of V49–P50, V69–P70 and V199–P200 as shown in Fig. 1. Purple membrane was isolated by the standard method as described in the literature [36] and was suspended in 5 mM HEPES buffer containing 0.02% NaN_3 and 10 mM NaCl at pH 7. These preparations were concentrated by centrifugation and placed in a 5 mm o.d. zirconia pencil-type rotor for magic angle spinning (MAS) experiments. Sample rotors were tightly closed by a teflon cap which was glued to the rotor by rapid Alardyte to prevent dehydration of the pelleted samples. Similarly, [2-^{13}C]Val-labeled bR was prepared. [1-^{13}C]Tyr-, [^{15}N]Pro-labeled bacterio-opsin (bO) was prepared by photo breaching of the corresponding bR in hydroxylamine solution (500 mM at pH 7) at 4 °C [24].

2.2. NMR measurements

High-resolution ^{13}C and ^{15}N NMR spectra were recorded by cross polarization-magic angle spinning (CP-MAS) on a Chemagnetics CMX-400 infinity FT-NMR spectrometer operated at 100.1 MHz for ^{13}C and 40.3 MHz for ^{15}N nuclei. The spinning frequency was set to 4 kHz, in order to avoid unnecessary heating as encountered for a fast MAS experiment, and probe temperature was kept to 20 °C by simply circulating cooled air. A ^{13}C, ^{15}N, and ^{1}H triple resonance MAS probe was used for REDOR filter experiments. The ^{13}C and ^{15}N chemical shifts were externally referred to 176.03 ppm and 11.59 ppm signals of carboxyl carbon and amino nitrogen of glycine with respect to TMS and NH_4NO_3, respectively. Conversion of ^{13}C chemical shift with respect to DSS, which is also utilized in solution NMR, can be made by adding 2.5 ppm to the data taken with reference to TMS [37,38].

^{13}C and ^{15}N spin–lattice relaxation times were measured using cross polarization and phase alternative saturation recovery pulse sequence [39,40]. Time intervals of 0.5, 1, 2, 3, 5, 7, and 10 s were measured for T_1 experiments. Spin–spin relaxation times under CP-MAS conditions were measured using spin echo pulse sequence by taking the pulse interval $\tau = N_c T_r$

$$M(2N_cT_r) = M(0)\ \exp\ (-2N_cT_r/T_2), \quad (1)$$

where N_c is number of rotor cycle between contact and π pulses and T_r is rotor period [41]. 2τ values of 2, 4, 6, 8, and 10 ms were used for T_2 experiments. ^{13}C- (or ^{15}N-) resolved, proton spin–lattice relaxation times in the rotating frame, ^{1}H $T_{1\rho}^{H}$, and cross relaxation times, T_{CH} (or T_{NH}), were evaluated by a nonlinear least-squares fit of the ^{13}C- (or ^{15}N-) peak-intensities $I(t)$ against the contact time t, from a stacked spectral plot of ordinary spectra as a function of the contact time.

$$I(t) = [I(0)/T_{CH}]\left[\exp\left(-t/T_{1\rho}^{H}\right) - \exp\ (-t/T_{CH})\right]\left[1/T_{CH} - 1/T_{1\rho}^{H}\right]^{-1} \quad (2)$$

where $I(0)$ denotes the initial peak intensity [42]. Contact times of 0.2, 0.5, 1, 2, 3, 4, and 5 ms were used to determine T_{CH} and $T_{1\rho}$ values.

3. Results

Fig. 2 shows the ^{13}C full echo (a), REDOR (b) and their difference spectrum as REDOR filter (c) spectra of [1-^{13}C]Val-, [^{15}N]Pro-bR from PM at the REDOR evolution time of 2 ms. Thus, we successfully resolved two ^{13}C NMR peaks at 171.1 and 172.1 ppm with the intensity ratio of 1:2 (higher field peak:lower field peak), corresponding to [1-^{13}C]Val signals of V49–P50, V69–P70 and V199–P200 pairs (see Fig. 1, Table 1).