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

Izuru Kawamura a, Masato Ohmine a, Junko Tanabe a, Satoru Tuzi b, Hazime Saitô c, Akira Naito a,⁎

a Graduate School of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan
b Graduate School of Science, University of Hyogo, Harima Science Garden City, Kamigori, Hyogo 678-1297, Japan
c Center for Quantum Life Sciences, Hiroshima University, Higashi-Hiroshima, Japan

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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 13C and 15N signals of [1-13C]Tyr-, [15N]Pro- and [1-13C]Val-, [15N]Pro-labeled bR. Rotational echo double resonance (REDOR) filter experiments were used to assign [1-13C]Val-, [15N]Pro signals to the specific residues in bR. The previous assignments of [1-13C]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 15N chemical shifts of Pro bonded to Val in the presence of 13C–15N correlation, although no assignment of peak is feasible for 13C nuclei not bonded to Pro. 13C–15N spin–lattice relaxation times (T1), spin–spin relaxation times under the condition of CP-MAS (T2), and cross relaxation times (TCH and TNH) for 13C and 15N nuclei and carbon or nitrogen-resolved, 1H spin–lattice relaxation times in the rotating frame (1H T1ρ) for the assigned signals were measured in [1-13C]Val-, [15N]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 13C and 15N conformation-dependent chemical shifts and T1, T2, 1H T1ρ, 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.

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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
* Corresponding author.
E-mail address: naito@ynu.ac.jp (A. Naito).

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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 13C solid state NMR. Fully hydrated bR in PM or reconstituted in lipid bilayer shows a variety of local motions in the order of 10⁶–10⁸ Hz, depending upon its particular sites, which may be related with the proton pump activity [18–20]. In particular, well-resolved 13C NMR signals are available from [3-13C]Ala-bR in PM, owing to their restricted motional frequencies in the transmembrane α-helices in the order of 10² 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 [3-13C]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, being recoupled 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 13C-15N pairs among those of 13C-15N 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 [35,36]. We have also observed that the cytoplasmic 15N peptide bonds as three 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 T2 experiments. Spin–spin relaxation times under CP-MAS conditions were measured using spin echo pulse sequence by taking the pulse interval τ = Nτc.

\[
M(2Nc,Tc) = M(0) \exp \left(-2NcT_2/T_2\right),
\]

where \(N_c\) is number of rotor cycle between contact \(\pi\) pulses and \(T_2\) is rotor period [41]. \(\tau\) values of 2, 4, 6, 8, and 10 ms were used for T2 experiments. 13C- (or 15N-) resolved spin–lattice relaxation times in the rotating frame, \(T_1^{\text{iso}}\), and cross relaxation times, \(T_{CH}^{\text{iso}}\) (or \(T_{CH}\)), were estimated by a nonlinear least-squares fit of the 13C- (or 15N-) peak-intensities \(I(t)\) against the contact time \(t\), from a stacked spectral plot of ordinary spectra as a function of the contact time. \(I(t) = [I(0)/T_{CH}] \left(\exp (-t/T_{1}^{\text{iso}}) - \exp (-t/T_{CH})\right) [1/\chi^{(2)} - 1/T_{1}^{\text{iso}}]^{-1}\) (2)

where \(T_{CH}\) 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 \(T_{1}^{\text{iso}}\) values.

3. Results

Fig. 2 shows the 13C full echo (a), REDOR (b) and their difference spectrum as REDOR filter (c) spectra of [1-13C]Val-, [15N]Pro-labeled bR against their NMR signals for the shorter FG and longer BC loops and helix B. In addition, we further examined the 15N and 13C NMR spectra of [1-13C]Tyr, [15N]Pro-labeled bacterio-opsin (bO) to clarify a manner of interaction of retinal with the extracellular side.
Contribution of background signals due to natural abundance (1.1%) carbonyl signals may be about 10%, because bR contains 248 amino acid residues. We further recorded the $^{13}$C CP-MAS NMR spectrum of [2-$^{13}$C]Val-labeled bR from PM, containing mainly two components of the transmembrane $\alpha$-helix (64.6 ppm) and $\beta$-sheet (56.4 ppm) with reference to those of 65.5 and 58.4 ppm, respectively[43,44], as an alternative means to characterize their local conformation, as shown in Fig. 3.

Fig. 4A shows the $^{15}$N CP-MAS NMR spectrum of [1-$^{13}$C]Val-, [15N]Pro-bR from PM. Five $^{15}$N signals of bR from PM taking 2D crystal were obviously resolved, free from background signals due to very low natural abundance (0.37%) between the 110 and 125 ppm region, which are resonated at the lower field than most of the amide $^{15}$N signals. It is noted that the $^{15}$N NMR peaks of [15N]Pro-bO were significantly suppressed (Fig. 4B) both at 114.5 and 122.4 ppm (Pro200 and Pro70 in the BC loop, respectively, as are assigned later) when retinal is removed from bR as compared with those of the corresponding bR (Fig. 4A, Table 1). In particular, the $^{15}$N NMR peak at 122.4 ppm present in bR was completely absent in bO. These results indicate that a large conformation change in bO is induced at least in the BC loop as a result of a local conformational change from the $\beta$-sheet to disorganized form.

Fig. 4C shows the $^{13}$C CP-MAS NMR spectrum of [1-$^{13}$C]Tyr-bR. Two major $^{13}$C peaks of bR from PM were observed at 174.0 and 176.0 ppm. Part of this doublet reflects the presence of two retinal configurations such as all-trans and 13-cis, and 15-syn forms, that coexist in the dark adapted state [27]. It was noticed that a broad single peak appeared in the $^{13}$C CP-MAS NMR spectrum of [1-$^{13}$C]Tyr-bO (Fig. 4D). This indicates that a large conformational change occurred and two protein conformations due to retinal isomers, disappeared.

Fig. 5 shows the $^{15}$N full echo (top) and REDOR filter (bottom) experiments of [1-$^{13}$C]Val-, [15N]Pro-bR. In the latter, the full echo spectrum is also reproduced in gray for the sake of comparison of peaks. The $^{15}$N resonances of the REDOR filter experiment at 111.4, 114.0, and 122.3 ppm decreased significantly from those of the full echo as indicated by the arrows, because of the presence of Pro residues corresponding to the consecutive Val-Pro sequence in bR (Fig. 1). In general, it has been shown that the $^{15}$N signal of the $\alpha$-helix form is resonated at higher field than that of the $\beta$-sheet form [45–47], as far as the $^{15}$N chemical shifts from the same amino acid residues are concerned. It is therefore possible to relate the $^{15}$N chemical shifts among different amino acid residues is not feasible, however, because the $^{15}$N chemical shift is also very sensitive to the presence of nearest neighbors [47].

The $^{13}$C and $^{15}$N spin–lattice relaxation times ($T_1$) and spin–spin relaxation times under CP-MAS conditions ($T_2$) for [1-$^{13}$C]Val-, [15N]Pro-bR were summarized in Tables 2 and 3, which were determined by cross polarization and phase alternative inversion recovery pulse sequence [39,40] and spin echo technique after cross polarization [41], respectively. The $^1$H $T_1^{\text{H}}$, and cross relaxation times, $T_{\text{CH}}$ (or $T_{\text{NH}}$) determined...
by analysis of cross polarization dynamics, were also included in Tables 2 and 3.

4. Discussion

4.1. $^{13}$C and $^{15}$N shifts of V49–P50, V69–P70 and V199–P200 pairs of [1-$^{13}$C]Val–, [15N]Pro-labeled bR

We assigned here the two $^{13}$C NMR peaks at 171.1 and 172.1 ppm of [1-$^{13}$C]Val–, [15N]Pro-bR to V199 and V49/69, respectively, on the basis of the REDOR filter experiment, as summarized in Table 1, although they were assigned to V69 and V49/69, respectively [48], using $^{13}$C SPECIFIC HETCOR experiment for Val-Pro pairs with an intensity ratio of 1:2. On the other hand, as pointed out already by our group, none of these peaks together with the peak at 172.9 ppm, mainly from the loop regions, could be ascribed to single carbons, as judged from comparative studies with site-directed mutants, in which decreased peak-intensities were seen from the site-directed mutants V49A, V101A, V130A and V199A [49–52]: the $^{13}$C NMR peaks at 172.9, 172.1, 171.1 ppm were assigned to V69/34, V49/130 and V101/199, respectively (Table 1). The 172.9 ppm peak was also assigned to V69 as viewed from a spectral change caused by chymotrypsin cleavage [50]. Nevertheless, the present finding indicates that the peak at 172.1 ppm can be ascribed to both V49/69 by the REDOR filter experiment (the intensity of the peak at 172.1 ppm is twice as large as that at 171.1 ppm), although superposition of the V130 peak cannot be confirmed by this sort of experiment [51]. The current assignment of the peaks at 172.1 to V69 based on the REDOR filter experiment seems to be more reliable than the previous one, although an additional assignment of the peak at 172.9 ppm to other Val residues besides V34, instead of V69, is not yet done at present, and the peak at 172.1 ppm can be unequivocally assigned to V49/69/130.

It appears that the assigned peaks at 172.1 ppm to V49 from [1-$^{13}$C]Val-residues is displaced substantially upfield from the $^{13}$C conformation-dependent chemical shifts of the $\alpha$-helix as a reference at 174.9 ppm [22,23,43,44]. This discrepancy may arise from the fact that the chemical shift of carbonyl carbon may be shifted to higher field by 1–2 ppm when the carbonyl carbon is directly bonded to Pro residue [53,54]. Because V69 is

![Fig. 2. $^{13}$C REDOR filter experiment of [1-$^{13}$C]Val–, and [15N]Pro-labeled bR. (A) Full echo spectrum. (B) REDOR spectrum. (C) Difference spectrum between Full echo and REDOR spectra. Chemical shift regions corresponding to the secondary structures are shown in the top part.](image)

![Fig. 3. $^{13}$C CP-MAS NMR spectrum of [2-$^{13}$C]Val-labeled bR. Chemical shift region corresponding to $\alpha$-helix, loop, and $\beta$-sheet structures are shown in the top part.](image)

Table 1

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<th>$^{13}$C ppm</th>
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<td>Val</td>
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<td>34</td>
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<td>49, 130</td>
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<td>199, 101</td>
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<td></td>
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<td>Method</td>
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<td>Ref.</td>
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<td>Site-directed mutants</td>
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bonded to P70, the expected chemical shifts of 172.1 ppm should be equivalent to 174.1 ppm ascribable to the α-helix. Since cryoelectron microscope and X-ray studies at low temperature show that the BC loop is involved in the β-sheet structure, V69 at 172.1 ppm may show the 13C chemical shifts corresponding to the β-sheet by taking into account the Pro effects. However, the 13C NMR peaks of [1-13C]Val-labeled bR seem to not show any signals at the peak-position of the secondary structure of β-sheet as shown in the top of Fig. 2. These results indicate that secondary structure in the vicinity of V69 may form a β-sheet structure as has been reported by X-ray diffraction studies [5].

Moreover, the 13C–15N 2D correlation spectra of the above-mentioned three components showed that the pairs of 13C carbonyl carbon and 15N amide chemical shifts are 171.1–114.0, 172.1–111.4 and 172.1–122.3 ppm (the 13C and 15N chemical shift values are recalculated based on our references) [48]. Because P70 is involved in the β-sheet as discussed already, their 15N peak should be resonated at the lower field than that of P200 as far as the 15N chemical shifts are concerned as stated above. Therefore, we found that three 15N NMR signals at 111.4, 114.0 and 122.3 ppm in the 15N CP-MAS NMR spectrum of [15N]Pro-bR are uniquely assigned to P50, P200, and P70, respectively. The previous assignment by Petkova et al. is not consistent with the well-known data of the relative peak-position of 15N chemical shifts.

4.2. Local dynamics determined by 13C and 15N relaxation times

Carbon spin–spin relaxation times ($T_2$) under CP-MAS conditions can provide motional information about the individual carbon or nitrogen sites of interest, in contrast to the case...
of proton spin–lattice relaxation times in the rotating frame ($T_1^p$), in which information on individual sites would be masked by the presence of rapid spin–spin process. In general, the $T_2$ values strongly depend on the frequency of proton decoupling and magic angle spinning, and are affected by interference between incoherent random motion and coherent decoupling and spinning frequencies. Therefore, the overall relaxation rate $1/T_2$ can be given by

$$1/T_2 = (1/T_2)^S + (1/T_2)^M_D + (1/T_2)^M_C.$$

Here, $(1/T_2)^S$ is the transverse component due to static C–H dipolar interactions, and $(1/T_2)^M_D$ and $(1/T_2)^M_C$ are the transverse components due to the fluctuation of dipolar and chemical shift interactions, respectively. The latter two terms are given as a function of the correlation time $\tau_c$ by

$$\frac{1}{(T_2)^M_D} = \sum (4\gamma_I^2\gamma_H^2\hbar^2/15\delta^3)(I+1)(\tau_c/(1 + \omega_I^2\tau_c^2))$$

(4)

$$\frac{1}{(T_2)^M_C} = (\omega_0^2\delta^2\eta^2/45)(\tau_c/(1 + 4\omega_0^2\tau_c^2) + 2\tau_c/(1 + \omega_0^2\tau_c^2))$$

(5)

Here, $\gamma_I$ and $\gamma_H$ are the gyromagnetic ratios of $I$ and $S$ nuclei, respectively, and $r$ is the internuclear distance between spins $I$ and $S$. $\omega_0$ and $\omega_I$ are the carbon resonance frequency and the amplitude of the proton decoupling RF field, respectively. $\omega_I$ is the rate of spinner rotation. $\delta$ is the chemical shift anisotropy and $\eta$ is the asymmetric parameter of the chemical shift tensor. They are affected by the frequency range of either 50 kHz ($\omega_0$) or 4 kHz ($\omega_I$), respectively. It is important to point out that the $(1/T_2)^S$ term dominates for a strongly coupled C–H system such as the methylene group, and therefore $T_2$ values are not sensitive for the group motion. On the other hand, carbonyl carbons do not have C–H bonds, and hence $(1/T_2)^M_D + (1/T_2)^M_C$ terms dominate the $1/T_2$. Consequently, $T_2$ values reflect the group motion. In this experiment, we measured $T_2$ values for carbonyl carbon and proline nitrogen whose nuclei do not bond to proton, and hence $T_2$ values are sensitive to the local motion. In summary, $T_1$, $T_2$ and $T_1^H$, values are sensitive in the motional frequencies of $10^8$ Hz, $10^4$–$10^5$, and $10^4$–$10^5$ Hz, respectively.

It is noted from the Tables 2 and 3 that the $^{13}C$ $T_1$ value of V199 (FG) and 101 (CD) (171.1 ppm) is the shortest among other signals, while the corresponding $T_2$ value is the longest. The $T_{CH}$ value of V199 is of the same order of magnitude as those of the others as the strength of the dipolar interaction is comparable to that of V49/69/130 at 172.1 ppm. This result indicates that the long FG loop as viewed from the $^{13}C$ NMR signal of V199/101 undergoes relatively rapid anisotropic fluctuations ($\sim 10^8$ Hz), although the short CD loop undergoes fluctuation motion with a frequency of $10^4$ Hz as viewed from suppressed peaks of [1–$^{13}$C]Ala-bR [25]. This motion is not sensitive to proton decoupling frequency ($10^4$ Hz) for a wild type bR as far as the $T_1$ value of theirs is concerned. It has been reported that the fluctuation frequency of the FG of bR is changed to lower frequency in the order of $10^6$–$10^4$ Hz for single and multiple mutants such as E194Q, E204Q, and E194Q/E204Q [50,55]. As E194 and E204 play important roles in the proton release process, it is noted that fast fluctuations of FG loop in the order of $10^8$ Hz may be correlated with proton release process to the extracellular side. The $T_1$ value at 172.1 ppm, ascribable to V49/69, on the other hand, exhibited the longest $T_1$ value, while the $T_2$ showed the intermediate value. These groups may contain components of slow motion ($10^4$ Hz) as judged from the $T_2$ values and $^{1}H$ $T_{1p}$ values, although this peak has been superimposed to V49/69 (helix B and BC loop). The $T_{CH}$ value at 172.9 ppm corresponding to V34 is the shortest among them, indicating that there is strong C–H dipolar interaction since it is located in the short AB loop (Fig. 1).

The $^{15}N$ NMR peaks of P50 and P70 were clearly resolved at 111.4 and 122.4 ppm from the other peaks (Fig. 4A) and their motional characteristics are straightforward on this relaxation behavior. This is not true for P200, because its $^{15}N$ signals are not well resolved. The $^{15}N$ $T_1$ values of P50 (111.4 ppm) and P70 (122.4 ppm) were significantly longer than those of the other peaks. On the other hand, the corresponding $^{15}N$ $T_2$ values exhibited the same order of magnitude as the other groups with the frequency of $10^4$ Hz. Therefore, these fluctuation frequencies of P50 (center of the helix B) and P70 (the BC loop) are in the same order of fluctuation as $10^6$–$10^8$ Hz. Surprisingly, it is noted that the P70 located in the long BC loop is as rigid as the P50 involved in the helix B. Thus it is reasonable to consider that the BC loop is involved in the $\beta$-sheet as judged from the conformation-dependent $^{13}C$ chemical shifts as described above and also shown by X-ray study, resulting in the reduced molecular motion in this group. In contrast, it is of interest to note that the $T_{NH}$ value of P70 was 1.12 ms and distinctly longer than the other four peaks. Nevertheless, these findings indicate that the BC loop in the vicinity of P70 takes large amplitude motions to average dipolar interaction to cause reduction of C–H dipolar interaction. In fact, the $T_{CH}$ value at 172.1 ppm involving V69 was also long, where V69 largely
dominate over 172.1 ppm in view of the dynamic state. It is, therefore, emphasized that local structure in the vicinity of V69–P70 in the BC loop forms a rigid β-sheet with large amplitude motions.

4.3. Dynamics change in bacterio-opsin

In our previous work, the β-sheet in bR reduced the thermal stability by cleaving bonds of 71–72 position [10]. This result indicates that the β-sheet in BC loop plays an important role for the proton transport activity. When retinal is reconstituted into bO, the characters of proton pump activity in regenerated bR are different from wild type bR [9,56]. Actually, the 15N NMR spectrum of regenerated bR did not show the same spectrum as the case of WT-bR. It is, therefore, noted that the dynamics in the BC loop may be related to the proton pump activity and stability of bR. This consideration proved that protein conformation is influenced from the cytoplasmic surface to the extracellular region by retinal isomerization through hydrogen bond networks.

The two major signals at 174.0 and 176.0 ppm were observed for [1-13C]Tyr-bR. In contrast, these two signals are not resolved on the 13C CP-MAS NMR spectrum of [1-13C]Tyr-bO due to the conformational disorder induced by the removal of retinal. Some of Tyr residues in bR are involved in hydrogen bond networks from retinal binding site to extracellular side [5]. These findings indicate that the hydrogen bond networks to the extracellular side are disordered by removal of retinal.

5. Conclusions

A variety of relaxation parameters which are sensitive to different frequencies for fluctuations were determined on the basis of the specifically assigned 13C and 15N NMR resonances, in order to characterize the dynamic features of the extracellular and cytoplasmic regions of bR. For this purpose, REDOR filter experiments were performed to confirm the assignment of 13C and 15N NMR signals of V69–P70, V49–P50 and V199–P200 in [1-13C]Val-, [15N]Pro-bR. In particular, the previous 13C NMR assignment to V69 was corrected on the basis of our newly assigned 15N chemical shift, by taking into account that the 15N chemical shift of the α-helix is resonated upfield than that of the β-sheet or loop. A variety of relaxation parameters (13C- and 15N-T1 and T2, TCH, TNN, 1H T1α) for the assigned peaks were determined for [1-13C]Val, [15N]Pro-bR. The 13C- and 15N-T1 values of V199/P200 indicate that the long FG loop has a fast fluctuational motion with the frequency of 108 Hz. On the other hand, 13C- and 15N-T2 of V69/P70 indicate that the BC loop of bR is involved in rigid β-sheet in spite of possessing large amplitude motions. This rigid β-sheet structure in the BC loop may play an important role for stabilizing the hydrogen bond networks in the extracellular region of the proton release group. Since the structure of the BC loop drastically changed by removal of retinal, it is evidenced that the BC loop may interact with retinal through hydrogen bond networks. This remote interaction may play an important role for proton release processes.

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