^{13}C NMR Study on Conformation and Dynamics of the Transmembrane α-Helices, Loops, and C-Terminus of [3-^{13}C]Ala-Labeled Bacteriorhodopsin[†]

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ABSTRACT: We have recorded ^{13}C CP-MAS and DD-MAS NMR spectra of untreated and deionized [3-^{13}C]-Ala-labeled bacteriorhodopsin (bR) and those cleaved with carboxypeptidase A and papain to gain insight into the conformation and dynamics of the transmembrane α-helices, loops, and C-terminus. It turned out that the C-terminus does not contribute to the ^{13}C CP-MAS NMR spectra of [3-^{13}C]Ala-bR recorded at ambient temperature owing to its rapid reorientational motions, since the relative peak intensities were unchanged in spite of the enzymatic cleavages. Therefore, the ^{13}C CP-MAS NMR peaks of bR should be ascribed both to the transmembrane α-helices and loops. We further distinguished the peaks of the α_{II}-helix form at 16.3 ppm (60%) from those of the α_I-helix form at 14.9 ppm (20%) by deconvolution of the respective peaks of the hydrated [3-^{13}C]Ala-bR, as referred to the ^{13}C chemical shift of polyalanine in hexafluoroisopropyl alcohol. The remaining CP-MAS NMR peak of [3-^{13}C]Ala-bR at 17.2 ppm was ascribed to the loops (20%) taking a variety of turn structures. In contrast, the ^{13}C NMR signals from the C-terminal residues were significantly enhanced by recording the dipolar-decoupled (DD)-MAS NMR spectra. Conformational features of the two different portions of the C-terminus, residues 245−248 and 231−244, were revealed by the conformation-dependent ^{13}C signals of bR successively cleaved by carboxypeptidase A and papain, respectively. The terminal end, residues 245−248, containing two Ala residues is virtually disordered and undergoing rapid motions. The inner segment (231−244) containing four Ala residues participates in an ordered conformation involving the α-helical conformation. This conformation is very stable with a lifetime much longer than 10^{-2} s, as viewed from the observed separation of sharp peaks.

Bacteriorhodopsin (bR)[1] is the sole protein present in the purple membrane (PM) of *Halobacterium halobium* which is active as the light-driven pump to translocate protons from the inside to the outside of the cell (Ovchinnikov, 1982; Stoeckenius & Bogomolni, 1982; Mathies et al., 1992). The recently determined model of bR based on electron and X-ray diffraction studies consists of seven transmembrane α-helices, shorter membraneous loops between the helices, and a retinal in the central pore of the α-helices (Henderson et al., 1990). It has been shown from the infrared, Raman, and CD spectra that the majority of the transmembrane α-helix is not the type of standard α-helix in which the amide planes are all nearly parallel to the helical axis (α_I-helix) but an α_{II}-helix in which the amide planes become significantly tilted with respect to the helical axis (Krimm & Dwivedi, 1982; Lee et al., 1987; Vogel & Gartner, 1987; Gibson & Cassim, 1989; Cladera et al., 1992). This view is consistent with our previous ^{13}C NMR study on [3-^{13}C]Ala-bR in which the major peak (16.3 ppm) in the hydrate state is significantly displaced downfield by 1.4 ppm from the α_I-helix as observed for polyalanine [(Ala)$_n$] (14.9 ppm) (Tuzi et al., 1993), although further experimental verification is required to assign this peak to the α_{II}-helix. The following three kinds of approaches are conceivable to this end. First, the conformation-dependent ^{13}C chemical shifts of Ala residue in the α_{II}-helix are available from recording ^{13}C NMR spectrum of (Ala)$_n$ dissolved in hexafluoroisopropyl alcohol (HFIP) solution (Krimm & Dwivedi, 1982). Second, the expected downfield displacement of Ala C_β ^{13}C peak due to a change of the torsion angles from the α_I- to α_{II}-helix can be now evaluated from the map of the ^{13}C shielding constant for *N*-acetyl-*N*′-methylalanine amide based on a theoretical calculation (Ando et al., 1984; Asakawa et al., 1994). Third, it is also useful to examine a plausible displacement of peaks, if any, by a conversion from the purple to blue membrane (the α_{II}- to α_I-helix) by deionization (Duñach et al., 1989).

The remaining 20 amino acid residues of bR at the C-terminus, however, have not being taken into account in the above-mentioned model, because the diffraction studies are not sensitive to presence or absence of the C-terminus owing to its disordered structure (Wallace & Henderson, 1982; Agard & Stroud, 1982). As to the dynamics of the C-terminus, the following two conflicting views have been demonstrated based on NMR and fluorescence studies: Keniry et al. (1984) demonstrated that the C-terminus is highly mobile on the time scale of ^{2}H NMR. This conclusion was later confirmed by high-resolultion ^{13}C NMR measurements (Bowers & Oldfield, 1988). However, neither ^{2}H NMR spectra of ^{2}H-labeled bR (Herzfeld et al., 1987) nor the [^{13}C]Leu-bR (Lewis et al., 1985) have revealed sharp spectral features which can be ascribed to isotropic motions.

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[1] Abbreviations; bR, bacteriorhodopsin; CD, circular dichroism; CP-MAS, cross polarization-magic angle spinning; DD-MAS, dipolar decoupled-magic angle spinning; FTIR, Fourier transform infrared spectroscopy; HFIP, hexafluoroisopropyl alcohol; PM, purple membrane; RH, relative humidity; TMS, tetramethylsilane.

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This finding is consistent with the data of Renthal et al. (1983), who demonstrated that the C-terminus is rigidly held at the membrane surface, on the basis of fluorescence study on dansylhydrazine attached to the C-terminal segments between 231 and 240. This view was opposed by Marque et al. (1986), who claimed that the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid attached to the C-terminus is free to assume many motions. Nevertheless, the role of the C-terminal tail is not clear at present, because it is not essential for proton-pump activity (Abdulaev et al., 1977), but the pump appears to work at a different rate without it (Govindjee et al., 1982).

Undoubtedly, NMR spectroscopy is a very suitable means to reveal conformation and dynamics of specific portions in the purple membrane, because ^{13}C chemical shifts are sensitively variable, reflecting local conformations of amino acid residues (Saitô, 1986; Saitô & Ando, 1989), and various types of NMR parameters are capable of detecting motions of a time scale from static to picoseconds (Bower & Oldfield, 1988; Zheng & Herzfeld, 1992), without leaving any ambiguity as to the labeled position and inevitable steric hindrance as in the cases of fluorescence probes. In practice, it is very important to clarify how the ^{13}C NMR signals from the C-terminus contribute to both the ^{13}C CP-MAS and DD-MAS NMR spectra of bR, because such a dynamic feature of the C-terminus, if any, may result in substantial change of the observed peak intensities obtained by the two methods. This information is essential for judging whether the previous assignment of peaks (Tuzi et al., 1993) needs an alteration for the C-terminus and loop regions or not.

In the present article, we aimed to clarify the previous assignment of the ^{13}C chemical shifts of [3-^{13}C]Ala-bR (Tuzi et al., 1993) ascribed to the secondary structures including those of the α_{II}-helix and loops in view of the above-mentioned conformation-dependent ^{13}C chemical shifts of (Ala)$_n$ in HFIP and spectral changes resulting from removal of the C-terminus utilizing enzymatically cleaved bR samples (Liao & Khorana, 1984) and deionization of PM sample (Duñach et al., 1987). Further, we were able to analyze the conformation and dynamics of the C-terminus of bR in a more specific manner in order to gain insight into the currently conflicting views based on the NMR and fluorescence data (Keniry et al., 1984; Lewis et al., 1985; Bowers & Oldfield, 1988; Herzfeld et al., 1987; Marque et al., 1986; Renthal et al., 1983).

MATERIALS AND METHODS

D,L-[3-^{13}C]Alanine was purchased from CIL, Cambridge, MA and used without further purification. All other amino acids were from Nakalai Tesque Chemical, Kyoto, Japan. Carboxypeptidase A (EC 3.4.17.1) and papain (EC 3.4.22.2) were purchased from Sigma Chemical Co., St. Louis, MO. *H. Halobium* strain S9 was grown in the TS medium of Ohnishi et al. (1965) in which an unlabeled L-alanine was replaced by [3-^{13}C]L-alanine. Purple membrane was isolated by the method of Oesterhelt and Stoeckenius (1973), followed by lyophilization from 2 mL of 10 mM sodium phosphate buffer (pH 7.0). Proteolysis with papain and carboxypeptidase A was carried out as described by Liao and Khorana (1984). Purple membrane (1.2 mg/mL) was incubated with cysteine-activated papain in 10 mM Tris buffer (pH 7.0) at a bR to papain ratio of 120:1 (w/w) at 37 °C for 3.5 h, or with carboxypeptidase A in 10 mM Tris buffer (pH 8.0) containing 0.15 M NaCl at a bR to enzyme ratio of 50:1 at 37 °C for 2.5 h. The digested purple membrane was washed with water and 10 mM sodium phosphate buffer (pH 7.0) and was then lyophilized from 2 mL of the buffer. The lyophilized purple membrane was hydrated by standing in an atmosphere of 100% relative humidity (RH) for 8−12 h in a desiccator or by addition of water (100% w/w). The specimens containing about 50 mg of the purple membrane were introduced into pencil rotors made of zirconia and tightly sealed with caps and glued with rapid Araldyte to prevent evaporation of water. Deionization of PM was performed by passage through a cation-exchange column (Dowex 50W) (Duñach et al., 1987).

Table 1: Conformation-Dependent ^{13}C Chemical Shifts of L-Alanine Residues

	solution NMR		solid-state NMR				
	α_{II}-helix	random coil	α_R-helix	α_L-helix	β-sheet	collagen	silk I
C$_\alpha$	53.2		52.4	49.1	48.2	48.3	50.5
C$_\beta$	15.8	17.2	14.9	14.9	19.9	17.6	16.6
C=O	178.4		176.4	172.9	171.8	173.1	177.1
reference	this work	*a*	*b*	*b*	*b*	*b*	*b*

[a] Saitô et al. (1982). [b] Saitô and Ando (1989).

High-resolution solid-state 100.7-MHz ^{13}C NMR spectra were recorded on a Chemagnetics CMX-400 NMR spectrometer by cross-polarization (CP) magic-angle spinning (MAS) and dipolar-decoupled (DD) MAS with a single pulse excitation methods. Measurements were carried out under dark condition. Ninety degree pulses for proton and carbon were both between 5 and 6 μs. Repetition time and contact time for the CP-MAS measurement were 4 s and 1 ms, respectively. The rotors were spun at 3.5∼4 kHz by compressed air. Transients were accumulated 2000−6000 times until a reasonable signal/noise ratio was achieved. ^{13}C chemical shifts were referred to the carboxyl signal of glycine (176.03 ppm from TMS) and then expressed as relative shifts from a value of tetramethylsilane (TMS). Resolution enhancement was carried out by the method of Gaussian multiplication (apodization time zero value of 0.8 and Gaussian broadening value of 50 Hz).

(Ala)$_n$ purchased from Sigma Chemical Co. (St. Louis, MO) was dissolved in HFIP, and its solution NMR spectra were recorded by a JEOL GX-400 NMR spectrometer. Chemical shifts were measured from the peak of benzene sealed in a capillary in this solvent and converted to the value from TMS.

RESULTS

First, we recorded the ^{13}C NMR spectrum of (Ala)$_n$ dissolved in HFIP, in order to obtain the conformation-dependent ^{13}C chemical shifts of Ala residue taking the α_{II}-helix conformation (Krimm & Dwivedi, 1982) as summarized in Table 1, together with the data of the other types of conformations (Saitô et al., 1982; Saitô & Ando, 1989). Obviously, the ^{13}C chemical shifts of the C$_\alpha$, C$_\beta$, and C=O signals of the α_{II}-helix form are significantly displaced from those taking another types of conformations and can be used as a good probe for the α_{II}-helix conformation. In particular, it is interesting to note that the carbonyl ^{13}C signal resonates at the lowest position, 178.4 ppm, being displaced downfield

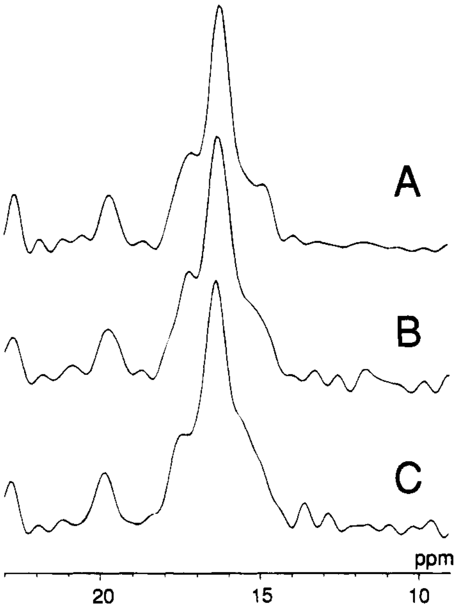

FIGURE 1: ^{13}C CP-MAS NMR spectra of untreated and cleaved [3-^{13}C]Ala-bR with proteolytic enzymes (100% RH): (A) untreated, (B) cleaved with carboxypeptidase A, and (C) cleaved by papain.

by 2 ppm from that of the standard α-helix of (Ala)$_n$ (176.4 ppm). This is consistent with the previous finding by Parrish and Blout (1972) that the carbonyl groups of (Ala)$_n$ in HFIP solution are more strongly hydrogen bonded than in a "classical" α-helix. They proposed an alternative α-helix structure [α_{II}-helix after Krimm and Dwivedi (1982)] for (Ala)$_n$ in HFIP solution which facilitates the formation of two hydrogen bonds with each carbonyl group (the doubly hydrogen-bonded helix), one hydrogen bond with a solvent hydroxyl and the other hydrogen bond with the peptide N−H group.

Figure 1A exhibits the ^{13}C CP-MAS NMR spectrum of Ala C$_\beta$ signals of the hydrated [3-^{13}C]Ala-bR equilibrated with 100% relative humidity (RH) at ambient temperature. The well-resolved ^{13}C chemical shifts of bR thus obtained are summarized in Table 2. It is noteworthy that a sharp peak at 17.0 ppm is strongly enhanced by the ^{13}C DD-MAS NMR spectrum of the same sample (Figure 2A). In contrast, there appears little change in the corresponding 16−18 ppm region of the CP-MAS spectra among untreated (Figure 1A), carboxypeptidase A-treated (Figures 1B), and papain-treated (Figure 1C) samples. We also recorded the corresponding spectral change by the ^{13}C DD-MAS NMR spectra of [3-^{13}C]-Ala-bR digested with carboxypeptidase A (Figure 2B) and papain (Figure 2C), respectively. It is emphasized that the 17.0 ppm peak disappeared from the ^{13}C DD-MAS NMR spectra of the specimen treated with the proteolytic enzymes (Figure 2B,C). The spectral changes could be seen more clearly by recording the difference spectra as shown in Figure 2: difference spectrum (D) between untreated (A) and carboxypeptidase A-treated (B); difference spectrum (E) between carboxypeptidase A-treated (B) and papain-treated (C). The peak position of the enhanced peak 17.0 ppm by the DD-MAS NMR differs significantly from the well-resolved peak at 17.2 ppm by the ^{13}C CP-MAS NMR spectrum (Figures 1 and 2). Closer examination at the 14−18 ppm region shows that the well-resolved peak observed at 14.9 ppm both in the CP-MAS (Figure 1A) and DD-MAS (Figure 2A) spectra of untreated [3-^{13}C]Ala-bR are changed into unresolved shoulder peak(s) spreading in this region after the proteolytic digestion with carboxypeptidase A and papain (Figures 1B,C and 2B,C). The peak intensity and position of the major peak at 16.3 ppm, however, were unaffected by the proteolysis.

Figure 3 demonstrates that the following three peaks at 17.8, 17.0, and 16.1 ppm were well resolved in the ^{13}C DD-MAS NMR spectra of [3-^{13}C]Ala-bR when the sample was fully hydrated by further addition of water (100% w/w). Here, it appears that the peak intensities of the ^{13}C signals due to the transmembrane α-helices and loops were significantly decreased for the samples hydrated with 100% w/w water. Similar type of suppressed ^{13}C NMR signals was previously noted for rhodopsin in lipid-bilayers by Sefcik et al. (1983): rotational diffusion of rhodopsin with $\tau_{\mathrm{R}} \approx 20$ μs could lead to random modulation of the ^{13}C−^{1}H dipolar interactions at frequencies comparable to $^1/_\tau \approx 50$ kHz, thereby defeating dipolar decoupling. These peaks were still visible even though the sample was treated with carboxypeptidase A (Figure 3B), in contrast to the case of the sample hydrated by 100% RH (Figure 2). The peak intensity at 17.0 ppm, however, is significantly suppressed after papain treatment (Figure 3C). The difference spectrum between the untreated (Figure 3A) and carboxypeptidase A-treated samples (Figure 3B) is shown in Figure 3D. It is obvious that the peak at 17.0 ppm (Figure 3D) and the peaks at 16.1, 17.0, and 17.8 ppm (Figure 3E) were removed by the respective proteolytic digestions.

Figure 4A illustrates the ^{13}C CP-MAS NMR spectrum of deionized bR. As demonstrated in the difference spectrum (Figure 4D) between the deionized (Figure 4B) and untreated bR (Figure 4C), there appears an appreciable change in the peak intensity at around 15.7 ppm and 16.8 ppm due to the conformational change by the deionization, although these altered peak positions are not in agreement with the peak of α_{I}-helix (14.9 ppm). As demonstrated in Figure 5, there appears an enhanced peak-intensity at 15.5 ppm of the deionized and fully hydrated bR with expense of the 17.8 ppm in the ^{13}C DD-MAS NMR spectrum, as compared with that of the untreated bR (Figure 3A).

DISCUSSION

The Assignment of Peaks to the Transmembrane α-Helices and Loops. In our previous paper (Tuzi et al., 1993), we assigned the two or three resolved Ala C$_\beta$ ^{13}C signals of bR at the peak area between 14.9 and 16.3 ppm to the transmembrane α-helices, although the major peak at 16.3 ppm is much displaced by 1.4 ppm from the standard Ala C$_\beta$ ^{13}C NMR signal of α-helix (14.9 ppm) (Saitô, 1986; Saitô & Ando, 1989). A similar type of anomaly has been observed by the previous studies with infrared spectroscopy: the amide I frequency (1660 cm^{-1}) and amide A frequency (3310 cm^{-1}) fall outside the normal range for an α-helix (1650−1655 and 3290−3300 cm^{-1}, respectively) (Rothschild et al., 1982). Krimm and Dwivedi (1982) ascribed, on the basis of the normal coordinate analysis of IR spectra, that the transmembrane α-helices of bR are of the α_{II}-type where amide planes are tilted from the helical axis. This view was later supported by the measurements of the circular dichroism of oriented film (Gibson & Cassim, 1989) and Raman spectroscopy (Vogel & Gartner, 1987). Therefore, it is more likely that the ^{13}C NMR peak at 16.3

Table 2: Assignments of ^{13}C NMR Peaks of Ala C_β in bR to Respective Conformations

		CP-MAS			DD-MAS			
	condition of hydration	α_{I}-helix	α_{II}-helix	loop	α-helix		random coil	loop
bR	100% RH	14.9	16.3	17.2 (17.5)^a			17.0	
	100% w/w				16.1		17.0	17.8
deionized bR	100% RH	14.8^b	16.1	17.1^b				
	100% w/w				16.0	16.3	17.0	

^{a} A peak observed for papain-treated bR. ^{b} A shoulder peak.

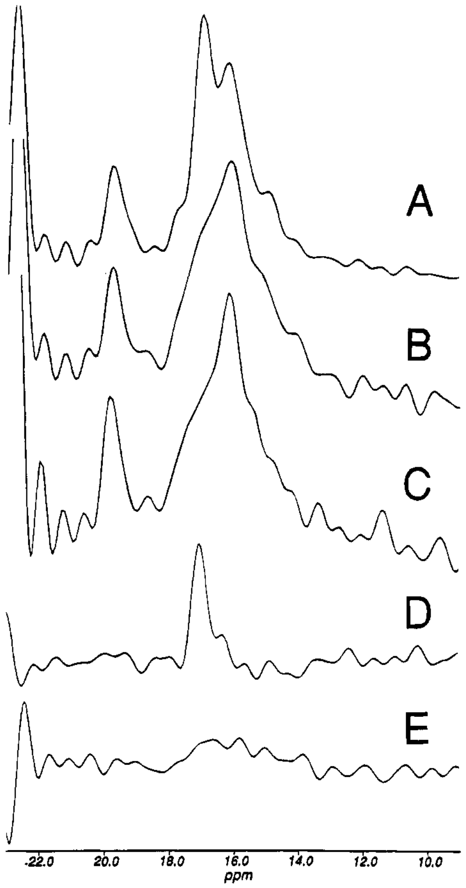

FIGURE 2: ^{13}C DD-MAS NMR spectra of untreated and cleaved [3-^{13}C]Ala-bR with proteolytic enzymes (100% RH) (A) untreated, (B) cleaved with carboxypeptidase A, (C) cleaved with papain, (D) difference spectrum between A and B, and (E) difference spectrum between B and C.

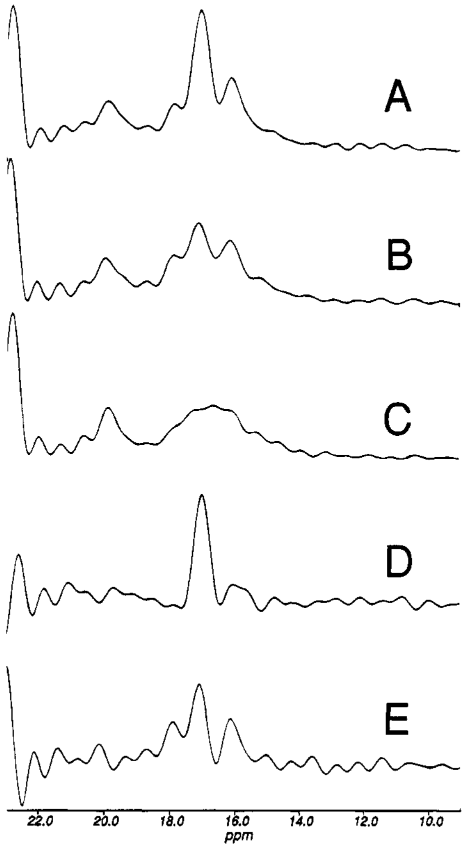

FIGURE 3: ^{13}C DD-MAS NMR spectra of untreated and cleaved [3-^{13}C]Ala-bR (hydrated by addition of 100% w/w water). (A) Untreated, (B) cleaved with carboxypeptidase A, (C) cleaved with papain, (D) difference spectrum between A and B, and (E) difference spectrum between B and C.

ppm arose from the α_{II}-helix, instead of the standard α_{I}-helix.

In fact, the major peak of [3-^{13}C]Ala-bR at 16.3 ppm is unequivocally ascribed to the α_{II}-helix form, because its peak position is very close to the Ala C_β peak of $(Ala)_n$ in HFIP solution (15.8 ppm) taking into account errors inherent in comparison of the ^{13}C chemical shifts between the solid and solution (Table 1). Krimm and Dwivedi (1982) proposed that plausible torsion angles (ϕ,φ) of the α_{II}-helix vary from (−96°, −16°) to (-70°, −36°), although the values (-65°, −40°) have been proposed for $(Ala)_n$ in HFIP (Parrish & Blout, 1972). Further, the expected change of the nuclear shielding constant arising from a conformational change from the α_{I}-helix to the α_{II}-helix is estimated as large as 1 ppm (downfield shift) depending on the exact torsion angles from the shielding map for Ala residue computed for *N*-acetyl-*N'*-methyl-L-alanine amide, using 4-31G ab initio basis set (Asakawa et al., 1994). The expected Ala C_β signal for the α_{II}-helix is thus 14.9−15.9 ppm and is consistent with the direction and magnitude of the displacement of the present experimental finding. Moreover, Duñach et al. (1989) demonstrated that deionization of purple membrane decreases the content of the distorted α_{II}-helices in favor of the more common α_{I}-helices, as judged from FTIR spectra. However, it turned out that this is not the case in our ^{13}C NMR study: the intensity of the peak at 14.9 ppm (α_{I}-helix) was not increased by the deionization, although the peak at around 15.7 ppm was enhanced to some extent by the deionization. It is worthwhile here to evaluate the relative proportion of Ala residues involved in the α_{II}- and α_{I}-helices on the basis of the deconvolution of the ^{13}C CP-MAS NMR spectrum of [3-^{13}C]Ala-bR into the four major Gaussian peaks (Figure 6). The relative peak intensities thus obtained are fairly in agreement with the data from the current model of the secondary structure (Table 3).

The well-resolved peak at 17.2 ppm recorded by the ^{13}C CP-MAS NMR spectra is obscured by overlap of another intense peak at 17.0 ppm recorded by the DD-MAS NMR spectra (Figures 1A and 2A). Any possibility that this signal

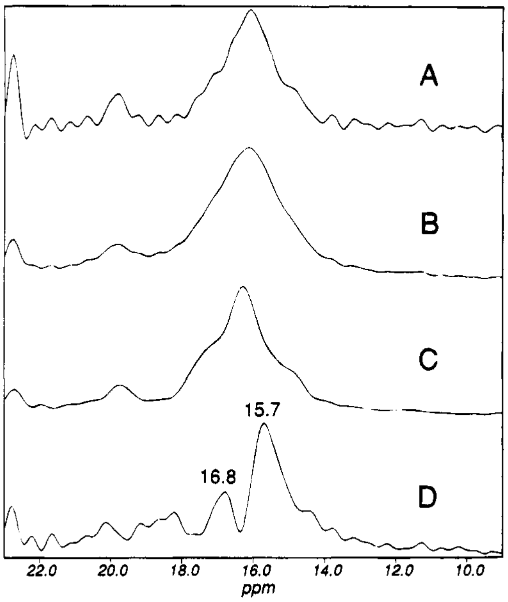

FIGURE 4: ^{13}C CP-MAS NMR spectra of deionized (A) resolution enhanced by gaussian multiplication, (B) without resolution enhancement, and untreated (C) without resolution enhancement [3-^{13}C]Ala-bR, and (D) difference spectrum between B and C (100% RH).

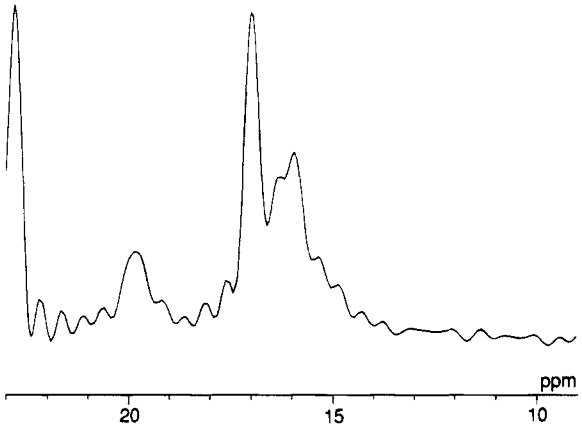

FIGURE 5: ^{13}C DD-MAS NMR spectrum of deionized [3-^{13}C]Ala-bR hydrated by addition of 100% w/w water.

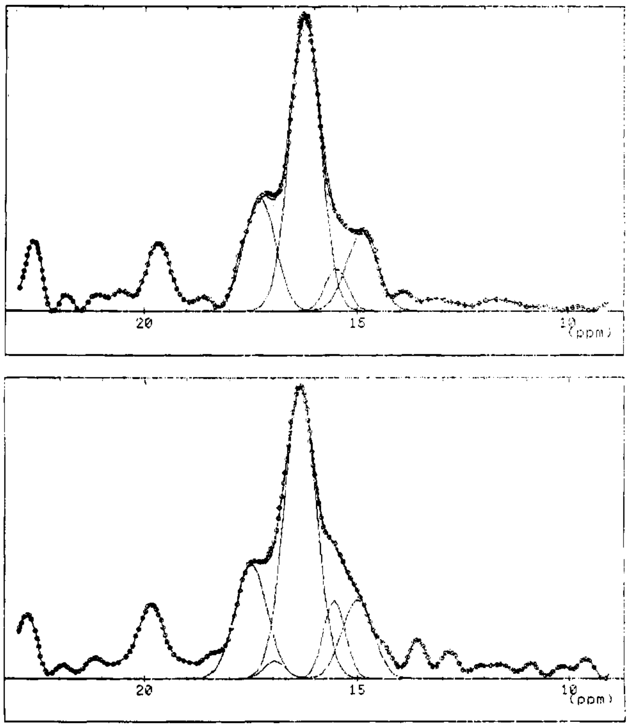

FIGURE 6: Deconvoluted ^{13}C CP-MAS NMR spectra of [3-^{13}C]-Ala-bR. (Top) Untreated bR (Figure 1A). (Bottom) Cleaved with papain (Figure 1D).

Table 3: Relative Intensities of Ala C_β Signals of bR in the CP-MAS Spectra Arising from the α-Helices, Loops, and N-Terminus

	loops + N-terminus (17.3 ppm)	α_{II}-helices (16.2 ppm)	α_I-helices (15.5 + 14.9 ppm)
relative peak intensity of Ala C_β in bR[a]	22%	57%	21%
relative numbers of Ala residues in model of bR[b]	17%	83%	

[a] The net peak intensity of Ala C_β in the CP-MAS NMR spectra of the [3-^{13}C]Ala-labeled bR in the hydrated PM was normalized to 100%. The spectra were recorded at 23 °C. [b] The number of Ala residues in the model proposed by Henderson et al. (1990) except residues in the C-terminus was normalized to 100%.

arose from the C-terminus is readily ruled out, because this signal remains unchanged even if the bR sample was digested either with carboxypeptidase A or papain (Figure 1B,C). Therefore, this peak should be ascribed to Ala residue(s) located in the loops connecting the α-helices or an N-terminus. It is true that the peak position 17.2 ppm (17.5 ppm in the case of the papain-treated bR; see Table 2) is very close to the value 17.0 ppm characteristic of the presence of the random coil conformation. However, it is premature to ascribe this signal to the random coil form, because this was made visible by the CP-MAS experiment. Vogel and Gärtner (1987) and Cladera et al. (1992) ascribed a considerable proportion of the amide I bands to the β-turn or reverse turns. It is thus likely that the peak at 17.2 ppm is ascribed to a variety of turn structures presented at the loop regions, although reference ^{13}C chemical shifts to confirm this postulate experimentally are not yet available.

As pointed out previously (Tuzi et al., 1993), the carbonyl peak of [1-^{13}C]Leu and Val-bR which resonates at 170–172 ppm was ascribed to the β-sheet form at the loops or termini. The presence of the β-sheet structure in bR has been pointed out from the measurements of FTIR, Raman, and CD spectra by Downer et al. (1986), Lee et al. (1987), Cladera et al. (1992), and Vogel and Gärtner (1987). In particular, Lee et al. (1987) showed that this β-structure is closely associated with the lipid bilayers and is not exposed to proteolytic digestion. It is unlikely, however, that Ala residue is involved in the β-sheet structure: First, the carbonyl peak of Ala C═O is resonated at the peak area between 177.6 and 173.6 ppm (Tuzi et al., 1993) the uppermost peak of which deviates by ca. 2 ppm from the standard β-sheet peak of $(Ala)_n$, 171.8 ppm (Saitô, 1986; Saitô & Ando, 1989). Second, we found that the peak 19.8 ppm which was previously assigned to the superposition of the β-sheet and lipid peaks disappeared when temperature was lowered below −60 °C (manuscript in preparation). It is unlikely that the β-sheet signal exhibits such an anomalous temperature-dependent change.

Conformation and Dynamics of the C-Terminus. The strongly enhanced 17.0 ppm peak only observable by the ^{13}C DD-MAS NMR spectra (Figure 2D) irrespective of the conditions for hydration, equilibrated with 100% RH or by addition of (100% w/w) water, is straightforwardly assigned to the terminal fragment (residues 231−248) of the C-terminus located in a cytoplasmic side of bR, because this signal disappears from the sample cleaved by carboxypeptidase A and papain (Figure 2). It appears that the terminal end of the anchored C-terminal tail would acquire motional freedom as in aqueous solution as far as the bR sample is fully hydrated and the residues under consideration are sufficiently separated from the membrane surface on which it is anchored. This is the reason why the ^{13}C signal from the C-terminus is visible only by ^{13}C DD-MAS NMR and disappears in CP-MAS NMR. In addition, it is noteworthy that the following three peaks, 17.0, 16.1, and 17.8 ppm, were well-resolved and the relative peak intensities with reference to the intensity at 14.9 ppm were enhanced when sufficient amount of water was added (100 %w/w) (Figure 3A). These peaks also disappeared when the specimen was treated with papain (Figure 2E). Therefore, they are ascribed to Ala residues in the C-terminus (residues 231−248). In particular, the single peak 17.0 ppm as observed in the difference spectrum (Figure 2D) is thus ascribed to two Ala residues at the C-terminus (residues 245 and 246) on the basis of the digestion profile by the two kinds of the proteolytic enzymes. These two Ala residues take disordered conformation as judged from the ^{13}C chemical shift data so far studied (Howarth & Lilley, 1978; Saitô et al, 1982).

On the contrary, all of the remaining four Ala residues which are located in the inner segment and cleaved by both papain and carboxypeptidase A do not always take random coil form. Instead, it appears that this portion takes a unique restricted conformation in which one Ala residue resonating at 16.1 ppm is involved in an α-helix, two Ala residues (at 17.0 ppm) are in a random coil, and the remaining one Ala residue (at 17.8 ppm) is in a form not yet specified but probably in a turn structure. This kind of secondary structure seems to be not unrealistic for this region, because an empirical prediction of a secondary structure by the parameters of Chou and Fasman (1978) showed that the residues 228−234 may be involved in the α-helix in which two Ala residues are located. In this connection, it is interesting to note that many short linear peptide fragments of proteins are able to adopt folded conformations in aqueous solutions (Wright et al., 1988; Dyson et al., 1991). For example, the C-peptide of ribonuclease A and the T cell stimulating peptide contain the α-helix and random coil structures, and the C-helix fragment of myohemerythrin contains the random coil and nascent helix which is a series of turn-like structures (Osterhout et al., 1989; Waltho et al., 1989; Dyson et al., 1988). It is well conceivable that this kind of secondary structure might be stabilized by the presence of a variety of metal ions. In fact, native purple membrane ($\lambda_{\max}$ 588 nm) contains one bound Ca^{2+} and four Mg^{2+} per bR molecule (Chang et al., 1985; Ariki & Lanyi, 1986). Removal of these cations thus causes a color transition from purple to blue (Chang et al., 1985; Kimura et al., 1984; Kobayashi et al., 1983). Further, it has been also shown that four to six low affinity Ca^{2+} binding sites are located at the C-terminus (Zhang & El-Sayed, 1993). As a candidate for this metal binding site, it is pointed out that two Glu and one Asp residues have acidic side chains in the segment digested by papain. Therefore, the obvious spectral change of the ^{13}C DD-MAS NMR spectra of the deionized bR (Figure 5) is well interpreted in terms of the conformational modification for the segment containing metal-binding sites as a result of removal of the metal ions. It is emphasized that the lifetime of this type of conformation should be longer than 10^{-2} s, as viewed from the observed separation of peaks.

The present finding implies that the conformation and dynamics of the C-terminus in bR differ substantially from portion to portion under consideration. This conclusion seems to be very reasonable because amplitude and motional frequency might vary depending upon the location relative to the anchoring position. Therefore, the previous fluorescence results by Marque et al. (1986) are well explained by the fact that the hydrophilic fluorescence probe is located at the terminal end and is able to detect free motions. On the contrary, the alternative view claiming unflexible feature of the C-terminus by Renthal et al. (1983) is consistent with our present ^{13}C NMR result if the probe molecule is located at the inner portion near the residues 231−244 as suggested by the authors. It is rather unlikely from this explanation, however, to conclude that many of surface region including loops are undergoing isotropic motions, as claimed by Kerniry et al. (1984) and Bowers and Oldfield (1988), because no Ala C_β ^{13}C NMR signals from these loop regions is enhanced by the DD-MAS experiments. However, it is also true that the ^{13}C peak intensity as recorded by the DD-MAS NMR strongly depends upon the condition of hydration (Figures 2 and 3).

Finally, it is interesting that the proteolytic cleavages at the C-terminus resulted in the increased peak intensities of the peak area at 15.0−15.5 ppm, as shown in Figure 1B,C. The deconvoluted spectrum (Figure 6, bottom) shows that the peak intensity at 15.3 ppm is significantly increased (from 5.7% to 9.8%). Similar type of spectral change was also noted in the ^{13}C DD-MAS NMR spectra (Figure 2B,C). These findings indicate that the partial or complete removal of the C-terminus resulted in an additional conformational change in the α_I-helix region. However, further work is necessary to obtain any conclusion from this finding.

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