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Backbone dynamics of membrane proteins in lipid bilayers: the effect of two-dimensional array formation as revealed by site-directed solid-state ^{13}C NMR studies on [3-^{13}C]Ala- and [1-^{13}C]Val-labeled bacteriorhodopsin

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Abstract

We have recorded site-directed solid-state ^{13}C NMR spectra of [3-^{13}C]Ala- and [1-^{13}C]Val-labeled bacteriorhodopsin (bR) as a typical membrane protein in lipid bilayers, to examine the effect of formation of two-dimensional (2D) lattice or array of the proteins toward backbone dynamics, to search the optimum condition to be able to record full ^{13}C NMR signals from whole area of proteins. Well-resolved ^{13}C NMR signals were recorded for monomeric [3-^{13}C]Ala-bR in egg phosphatidylcholine (PC) bilayer at ambient temperature, although several ^{13}C NMR signals from the loops and transmembrane α-helices were still suppressed. This is because monomeric bR reconstituted into egg PC, dimyristoylphosphatidylcholine (DMPC) or dipalmytoylphosphatidylcholine (DPPC) bilayers undergoes conformational fluctuations with frequency in the order of 10^4–10^5 Hz at ambient temperature, which is interfered with frequency of magic angle spinning or proton decoupling. It turned out, however, that the ^{13}C NMR signals of purple membrane (PM) were almost fully recovered in gel phase lipids of DMPC or DPPC bilayers at around 0 °C. This finding is interpreted in terms of aggregation of bR in DMPC or DPPC bilayers to 2D hexagonal array in the presence of endogenous lipids at low temperature, resulting in favorable backbone dynamics for ^{13}C NMR observation. It is therefore concluded that [3-^{13}C]Ala-bR reconstituted in egg PC, DMPC or DPPC bilayers at ambient temperature, or [3-^{13}C]Ala- and [1-^{13}C]Val-bR at low temperature gave rise to well-resolved ^{13}C NMR signals, although they are not always completely the same as those of 2D hexagonal lattice from PM.

Keywords: Site-directed solid-state ^{13}C NMR; Membrane protein; Bacteriorhodopsin; Backbone dynamics; 2D hexagonal array

1. Introduction

It is well recognized that membrane proteins in the membrane environment are known to assemble into oligomeric complex as structural units rather than monomers in two-dimensional (2D) crystals as a result of forming tertiary and quaternary structures believed to be necessary for biological signaling [1,2]. This view has been confirmed by the revealed three-dimensional (3D) structures by cryoelectron microscope or X-ray diffraction for a number of membrane proteins either from 2D or 3D crystals, respectively [3–15]. In particular, proton pump bacteriorhodopsin (bR) from purple membrane (PM) of *Halobacterium salinarum* consists of naturally occurring 2D crystalline patches in which trimeric units of bR are hexagonally packed to form PM under physiological conditions [16]. It is interesting to note that 3D crystals of bR grown from cubic phase are also arranged in the hexagonal lattice with the same unit cell as that of 2D crystals [17,18]. The native structure of the trimeric unit is also preserved for bR from another type of crystalline structure consisting of honeycomb lattice grown from PM sheets, irrespective of alterations in the in-plane

Abbreviations: bO, bacterioopsin; bR, bacteriorhodopsin; CP-MAS, cross polarization-magic angle spinning; 2D, two-dimensional; DD-MAS, single pulse excitation with dipolar decoupled-magic angle spinning; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmytoylphosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; *pp*R, *pharaonis* phoborhodopsin; PM, purple membrane

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orientation of the trimer [7]. It has been pointed out that selective interactions between bR and certain endogenous lipid molecules [19,20], together with protein–protein interactions, are essential for lattice assembly [21].

It is anticipated, however, that oligomerization as well as lattice formation is not always straightforward for a number of membrane proteins overexpressed in a host cell like *E. coli*, unless otherwise identification and incorporation of such endogenous lipids to promote formation of 2D lattice are seriously taken into account, in view of the manner of 2D array studied for bR integrated into lipid bilayers [19–25]. In this connection, it was shown that the tetrameric arrangement of the chloride pump halorhodopsin in 2D crystal [8] is not retained in 3D crystal prepared from cubic phase system, but trimeric complex is formed instead [9]. In a similar manner, *pharaonis* phoborhodopsin (sensory rhodopsin II) is shown to be present as dimeric form in 2D crystal [10] but as the trimeric form in 3D crystals prepared from cubic phase system [11]. It is also worthwhile to point out that the functional unit responsible for bR's photocycle is the monomer itself [26–28], except that the life times of the L and N intermediates are significantly shorter in the monomer [29,30]. In fact, the light–dark adaptation is affected by the aggregation state of bR [27] and an increase in the relative amplitude of the slow component of bR excited state decay is observed in the monomer, which is due to the increase in the concentration of the 13-*cis* retinal isomer in the ground state of the light-adapted bR [31]. It is therefore expected that such oligomerization may play an important role in regulating the conformation and dynamics of individual protein molecules to express their functions.

It is demonstrated here that bR from PM as naturally occurring 2D crystals provides one the best environment at ambient temperature to be able to yield well-resolved ^{13}C NMR signals from bR labeled with [3-^{13}C]Ala, [1-^{13}C]Val, or other amino acid residues [32–37]. The site-directed ^{13}C solid-state NMR approach thus available turned out to be particularly useful means to reveal conformation and dynamics of such membrane proteins, once their well-resolved ^{13}C NMR peaks were assigned to particular residues by a site-directed manner, on the basis of the conformation-dependent displacement of ^{13}C NMR peaks with reference to the accumulated data base, comparative peak-intensities between wild-type and site-directed mutants involving the residue under consideration, cleavage by proteolytic enzymes, enhanced spin-relaxation times by surface-bound paramagnetic ions, etc. [32–38]. It is also emphasized that this particular means is useful to reveal conformation and dynamics for a number of membrane proteins, irrespective of the presence as monomer or oligomerized form as far as ^{13}C NMR spectra yielding full signal strengths are available as reference. In fact, we have so far assigned the ^{13}C NMR

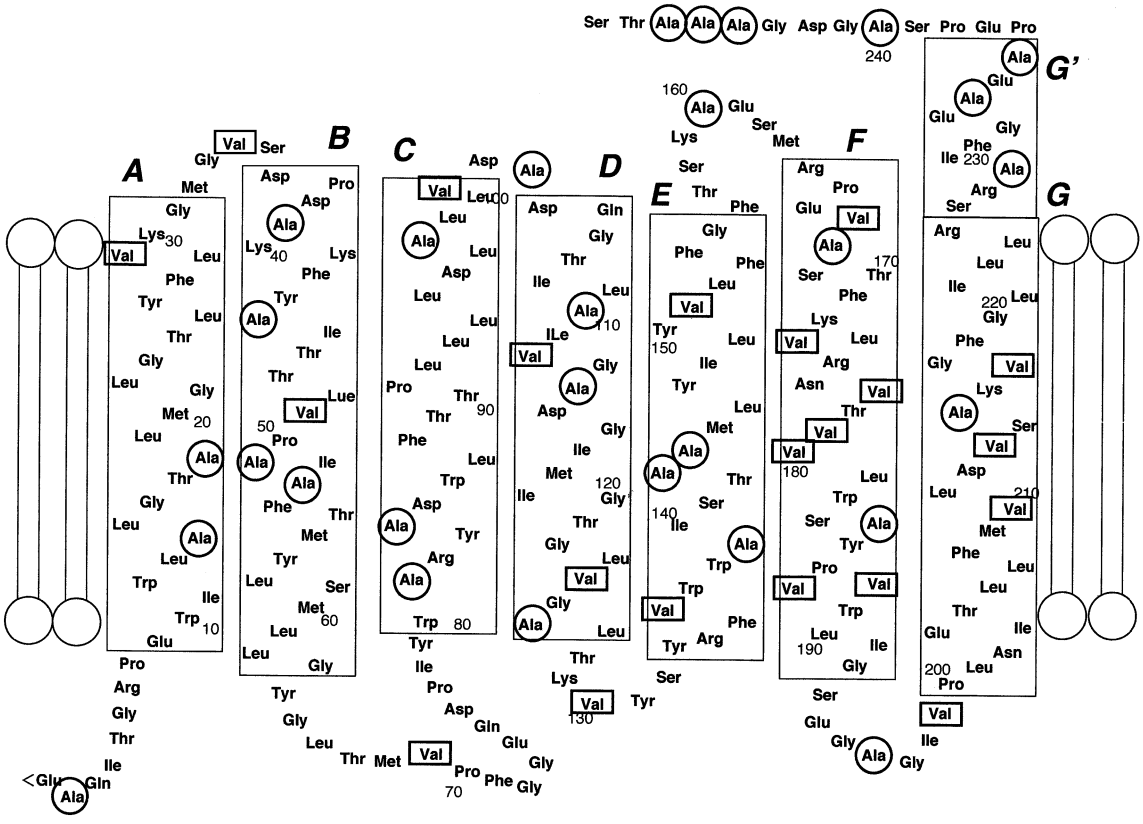

Fig. 1. Schematic representation of the secondary structure of bR taking into account its secondary structure revealed by X-ray diffraction of 3D crystals (Ref. [17]). The location of the C-terminal α-helix protruding from the membrane surface is indicated as G′ helix, together with the seven transmembrane α-helices indicated by the rectangular boxes, A–G. All the circled and boxed residues are shown by ^{13}C-labeled Ala and Val residues, respectively.

peaks of up to 60% residues of [3-^{13}C]Ala- and [1-^{13}C]Val-labeled bR whose labeled positions are indicated as circles and boxes, respectively, in its primary sequence taking into account the secondary structure revealed by X-ray diffraction of 3D crystals [17], as shown in Fig. 1 and Table 1 [32–38]. Nevertheless, it turned out that ^{13}C NMR spectra of [3-^{13}C]Ala-labeled W80L and W12L mutants of bR were substantially broadened together with the absence of ^{13}C NMR signals from the loop region, and ^{13}C NMR signals from [1-^{13}C]Val-labeled these mutants were completely suppressed [35]. Further, relative peak-intensities of surrounding lipids were substantially increased (100–200 lipids per protein) as compared with 10 lipids per protein in the wild-type bR present in the 2D crystalline lattice. This is caused by disrupted or disorganized 2D crystalline lattice structures and resulting induced fluctuation motions of protein backbone in the time scale of 10^{-4} to 10^{-5} s, resulting in failure of attempted peak-narrowing to yield high-resolution solid-state NMR [35].

In this connection, it is rather surprising to note that well-resolved ^{13}C NMR signals were recorded for [3-^{13}C]Ala and [1-^{13}C]Val-labeled phoborhodopsin (sensory rhodopsin II) integrated into egg PC bilayer, although ^{13}C NMR signals from the loop region are still suppressed [39]. These observations indicate that the expected spectral resolution of ^{13}C NMR spectra is strongly depending upon the manner of lipid–protein interaction to determine the resulting correlation times of fluctuation motions. For this reason, it seems to be very important to examine how ^{13}C NMR spectra of [3-^{13}C]Ala and [1-^{13}C]Val-bR in neutral lipid bilayers such as egg phosphatidylcholine (PC), dimyristoylphosphatidylcholine (DMPC) or dipalmytoylphosphatidylcholine (DPPC) will be modified in the presence of endogeneous lipids which are believed to play an essential role for lattice formation [19,20]. Thus, we aimed, in this paper, to record site-directed ^{13}C NMR spectra of [3-^{13}C]Ala, [1-^{13}C]Val-labeled bR incorporated into egg PC, DMPC or DPPC bilayers, in order to clarify how their spectra were modified by the presence or absence of the 2D crystalline lattice in bR. Further, we also examined how ^{13}C NMR spectra of bR monomers are varied at low temperature as a result of the aggregation of the proteins to form 2D array. We found that ^{13}C NMR spectral pattern of 2D array of bR which is similar to that of 2D lattice is obtained in the presence of endogenous lipids at low temperature as in the gel phase state of DMPC or DPPC bilayer.

Table 1
Assigned ^{13}C chemical shifts for [3-^{13}C]Ala- and [1-^{13}C]Val-labeled bacteriorhodopsin (ppm from TMS)

		Ala	Val
[3-^{13}C]Ala[a]	17.78	196	
	17.36	160	
	17.27	184	
	17.19	103, 235	
	16.88	84, 240, 244–246	
	16.38	39, 168	
	16.52	81	
	16.20	215	
	16.14	53	
	15.92	51	
	15.91–15.67	228, 233	
	15.02	126	
[1-^{13}C]Val[b]	171.07		101, 199
	171.99		49, 130
	172.84		34, 69
	173.97		151, 167, 180
	174.60		136, 179, 187
	174.97		217
	177.04		29, 213

[a] From Ref. [34].
[b] From Ref. [37].

2. Materials and method

L-[3-^{13}C]Alanine and L-[1-^{13}C]valine were purchased from CIL, MA, Andover, USA and used without further purification. *H. salinarum* S9 was grown in TS medium of Ohnishi et al. [40], in which an unlabeled L-alanine and L-valine were replaced with [3-^{13}C]alanine and [1-^{13}C]valine, respectively. bR was isolated as PMs by the method of Oesterhelt and Stoeckenius [41]. Experimental process at this stage was done at 4 °C. bR was pelleted by centrifugation (40 000 × *g*, 4 °C, 60 min) from its suspension in 10 mM HEPES, 10 mM NaCl, 0.025% (w/v) NaN_3 at pH 7. The resulting pellet was resuspended on high-purity water (resistivity 18 MΩ cm) and centrifuged to remove the HEPES buffer and solubilized by stirring in 5% (w/v) DM (bR: DM = 1:1280 (mol/mol) = 1:25 (w/w)) containing 20 mM MES, 0.025% (w/v) NaN_3 at pH 5 after sonication for 30 s and left at 42 °C for 10 days in the dark until a clear solution was obtained. The solubilized bR thus obtained was mixed with multibilayers of egg PC, DMPC or DPPC and dialyzed against buffer solution containing 5 mM MES, 10 mM NaCl, 0.025% NaN_3 at pH 7 for 10 days, at temperatures of 5, 25 or 42 °C, respectively, depending upon the variety of lipids by taking into account the phase transition temperatures of the individual lipids. Buffer solution was changed twice a day for the first few days. The resulting preparation was incubated with Bio-Beads for 20 h at 4, 20 and 42 °C depending on the systems of egg PC, DMPC and DPPC bilayers, respectively. Absorption spectra were always checked immediately after each experimental step. Protein to lipid ratio was kept to 1:50 (mol/mol) throughout the experiment. The pellets thus obtained were placed into a 5 mm o.d. zirconia pencil-type rotor. The caps were tightly glued to the rotor by rapid Araldite® (Vantico) to prevent leakage of water from the samples during magic angle spinning for NMR measurements.

^{13}C NMR (100.63 MHz) of fully hydrated pelleted preparations of [3-^{13}C], [1-^{13}C]Val-bR spectra were recorded in the dark at 20 °C on a Chemagnetics CMX 400 spectrometer both by cross polarization-magic angle

spinning (CP-MAS) and single pulse excitation with dipolar decoupled-magic angle spinning (DD-MAS) method to distinguish ^{13}C NMR signals of more flexible membrane surface from those of rigid transmembrane α-helices and loops [32,33]. The spectral width, contact time and acquisition time for CP-MAS experiments were 40 kHz, 1 and 50 ms, respectively. The 90° pulses for carbon and proton nuclei (5 μs each) and 45° pulse for carbon nuclei were used for the CP-MAS and DD-MAS experiments, respectively, together with the spinning rate of 4 kHz. Repetition times for the former and latter experiments were 4 and 6 s, respectively. Free induction decays were acquired with data points of 2000. Fourier transform was carried out as 16 000 points after 14 000 points were zero-filled to improve digital spectral resolution. ^{13}C chemical shifts were initially referred to the carboxyl carbon signal of glycine (176.03 ppm from tetramethylsilane (TMS)) and converted to the value relative to TMS.

3. Results

First of all, bR turns out to be very stable in spite of its exposure to environment at a temperature of 42 °C for 10 days during solubilization, and additional dialysis at 4, 20 and 42 °C for 10 days in the presence of egg PC, DMPC, and DPPC, respectively, as judged from unchanged UV absorption maximum at 560 nm for solubilized and reconstituted preparations in these lipid bilayers, with reference to the absorption maximum of 568 nm for intact bR (spectra not shown). The abovementioned spectral change was noted for a variety of delipidated and solubilized bR [42]. It is also pointed out that such intactness of bR preparation was also manifested from the similarity of ^{13}C NMR spectra among a variety of reconstituted systems, to be described later.

Fig. 2 illustrates the ^{13}C CP-MAS (A) and DD-MAS (B) NMR spectra of [3-^{13}C]Ala-labeled bR integrated into egg PC bilayer (protein to egg PC mole ratio of 1:50) at ambient temperature (20 °C) with reference to those of native PM (C and D). Notably, the intense peak at 16.8 ppm ascribable to Ala 228 and 233 located at the C-terminal α-helix protruded from the membrane surface ([32,34]; see Table 1 and helix G′ in Fig. 1) is clearly visible in the DD-MAS NMR (Fig. 2B) but substantially suppressed in the CP-MAS NMR (Fig. 2A). This is because ^{13}C NMR signals of [3-^{13}C]Ala-bR with sufficiently shorter spin-lattice relaxation times in the order of 0.5 s [44] as compared with the repetition times (6 s) are fully made visible in the DD-MAS spectra, although most signals from the flexible portion, such as the C-terminal residues, are suppressed in the CP-MAS spectra because of insufficient cross polarization. In addition, the ^{13}C NMR signals from the reconstituted preparation are resonated at the region of 14.5–17.1 ppm (Fig. 2A), with reference to the data of intact bR (Fig. 2C).

The abovementioned spectral range of the ^{13}C NMR spectra of the reconstituted bR in egg PC bilayer is slightly different from that of native PM resonated at region between 14.8 and 17.8 ppm (Fig. 2C). In particular, the lowermost two peaks arising from native bR, 17.8 (Ala 196 in F–G loop) and 17.4 ppm (Ala 160 in E–F loop) (see Table 1 and Fig. 1), are absent in the ^{13}C CP-MAS NMR spectra of

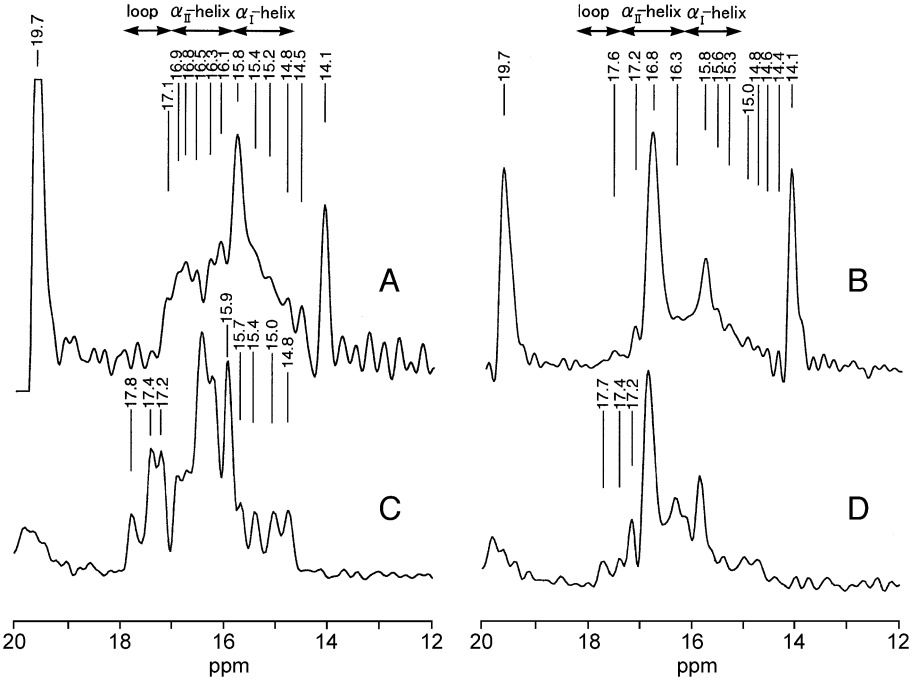

Fig. 2. ^{13}C CP-MAS (left) and DD-MAS (right) NMR spectra of [3-^{13}C]Ala-labeled bR reconstituted into egg PC bilayer (A and B; 1:50 mol ratio) and from PM (C and D). The intense peaks at 19.7 and 14.1 ppm are ascribed to lipid methyl groups from *Halobacteria* and egg PC, respectively.

reconstituted bR in egg PC at ambient temperature (Fig. 2A). The spectral range of the ^{13}C NMR peaks from bR in egg PC is also very similar to that of phoborhodopsin in egg PC [39] and also W80L or W12L mutants in the native PM lipids [35]. They seem to be common among a variety of membrane proteins in the absence or disorganized trimeric form [3] due to modified helix–helix or helix–lipid interactions caused by the site-directed mutation at Trp 80 and Trp 12. Further, it is notable that the ^{13}C NMR peak of the cytoplasmic C-terminal α-helix (helix G′) protruded from the membrane surface (Ala 228 and 233; Refs. [32–34]) resonated at 15.9 ppm in the intact PM (Table 1) is displaced upfield by 0.1–15.8 ppm in this preparation. These spectral changes are also noted in the DD-MAS NMR spectra (Fig. 2B and D). In addition, the intense lowermost peak at 19.7 ppm in the top traces arises obviously from the endogenous PM (methyl group) whose ^{13}C labeled nuclei were biosynthetically transferred from incorporated [3-^{13}C]Ala label and strongly bound to bR [35]. Naturally, the intense peak at 14.1 ppm is obviously ascribed to the lipid methyl group from egg PC in view of its absence in the native PM (C and D) [38].

Surprisingly, very broad and less-intense ^{13}C CP-MAS (A) and DD-MAS (B) NMR spectra were recorded from [1-^{13}C]Val-labeled bR in egg PC bilayer (Fig. 3) as compared with those of PM (C and D). In contrast to the case of [3-^{13}C]Ala-bR with shorter ^{13}C spin-lattice relaxation times (T_1 values), major ^{13}C NMR signals of [1-^{13}C]Val-bR by DD-MAS NMR are naturally suppressed because of the

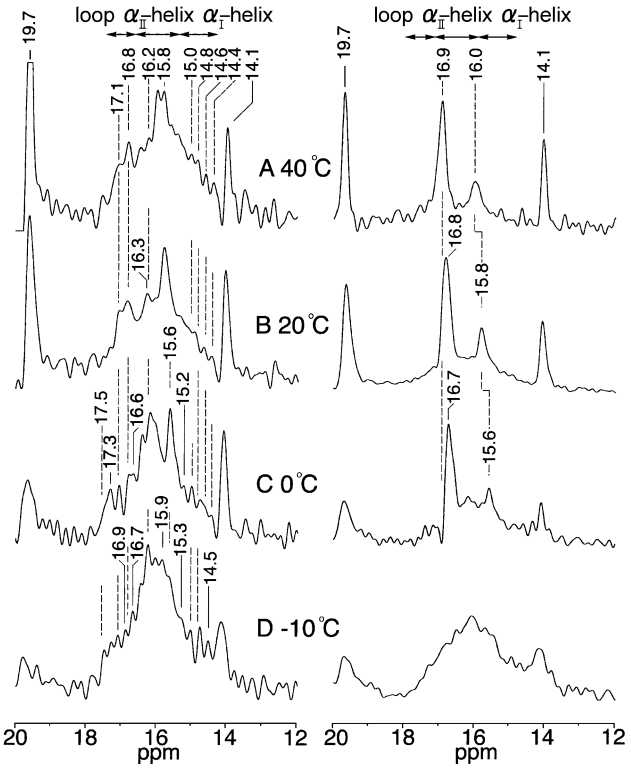

Fig. 4. ^{13}C CP-MAS (left) and DD-MAS (right) NMR spectra of [3-^{13}C]Ala-labeled bR reconstituted into DMPC bilayer (1:50 mol ratio) at various temperatures from 40 °C (A) to −10 °C (D).

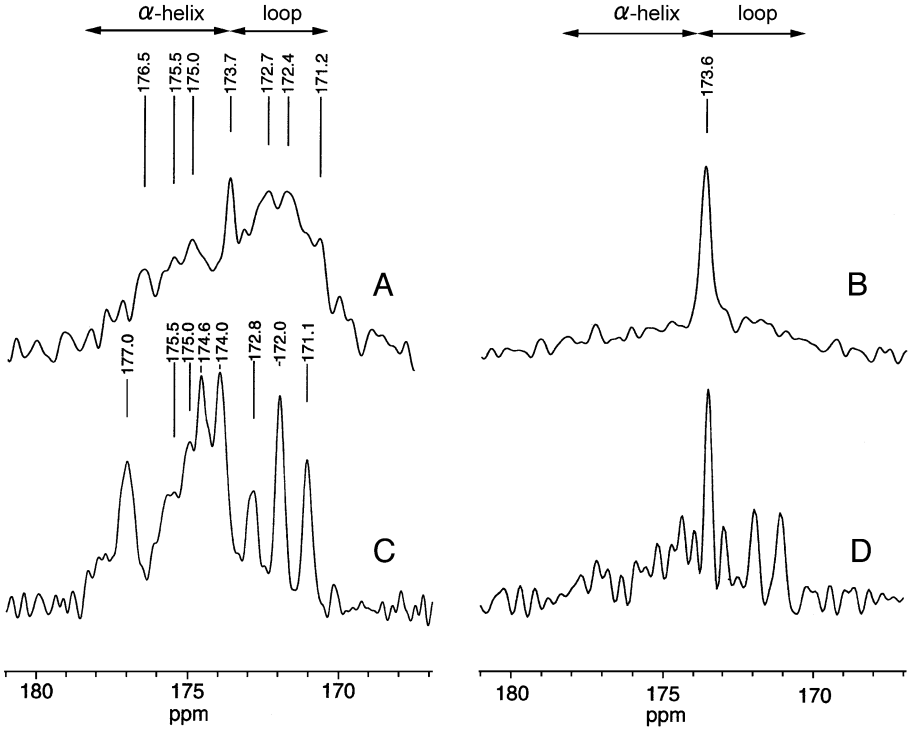

Fig. 3. ^{13}C CP-MAS (left) and DD-MAS (right) NMR spectra of [1-^{13}C]Val-labeled bR reconstituted into egg PC bilayer (A and B) and from PM (C and D).

longer ^{13}C T_1 values for the transmembrane helices (15–20 s) [44] as compared with the repetition time of 6 s. In this connection, the rather sharp signal resonated at 173.7 ppm (A), however, is not present in the native PM (C) and is readily ascribed to the carbonyl ^{13}C signals from lipid molecules instead of a peak from the protein. It is also notable that the manner of peak-suppression is more pronounced for the α-helical region than that of the loop region resonated at upper field (Val 69, 101, 130 and 198 from Table 1 [33,37], although Val 49 located at the transmembrane α-helix B is accidentally resonated at this region because of the presence of Pro 50. The substantially suppressed ^{13}C NMR signals of reconstituted [1-^{13}C]Val-bR in egg PC recorded by CP-MAS NMR were obviously caused by an insufficient peak-narrowing process due to interference of the fluctuation frequency of monomeric bR with the frequency of magic angle spinning [46]. Such differential peak-suppression seems to be caused by the magnitude of the respective chemical shift anisotropies which vary with the extent of plausible molecular motions among the α-helix and loop region [33,44,47]. It is emphasized, however, that the presence of such motions, if any, does not affect the ^{13}C NMR spectral feature of [3-^{13}C]Ala-labeled bR which is sensitive to frequency in the order of 10^5 Hz, rather than 10^4 Hz, corresponding to proton decoupling frequency [45].

It is expected that these ^{13}C NMR signals could be strongly influenced by the phase of used lipids and the temperature of the reconstituted preparations. Therefore, it seems to be more preferable to examine ^{13}C NMR spectra of bR integrated into DMPC or DPPC bilayer in which phase transition temperatures are well defined. In Fig. 4, we have illustrated the ^{13}C CP-MAS (left) and DD-MAS (right) NMR spectra of [3-^{13}C]Ala-labeled bR in DMPC bilayer at temperatures between −10 and 40 °C. It is pointed out that the ^{13}C NMR signals of [3-^{13}C]Ala-bR are very similar among preparations reconstituted in bilayers of egg PC (Fig. 2A), DMPC (Fig. 4B) and DPPC to be described later. This means that no change in the secondary structure of the protein was induced during dialysis at temperatures between 4 and 42 °C. In contrast to the ^{13}C NMR spectral feature observed at ambient temperature, well-resolved ^{13}C NMR signals are recorded at a temperature below zero. Further, several missing peaks (17.5 and 17.3 ppm) or peaks of reduced peak-intensities (16.7–16.0 ppm), including Ala 53, 215, 81, 39, 168, 103 and 235 (see Table 1 and also Fig. 1) both in egg PC and DMPC

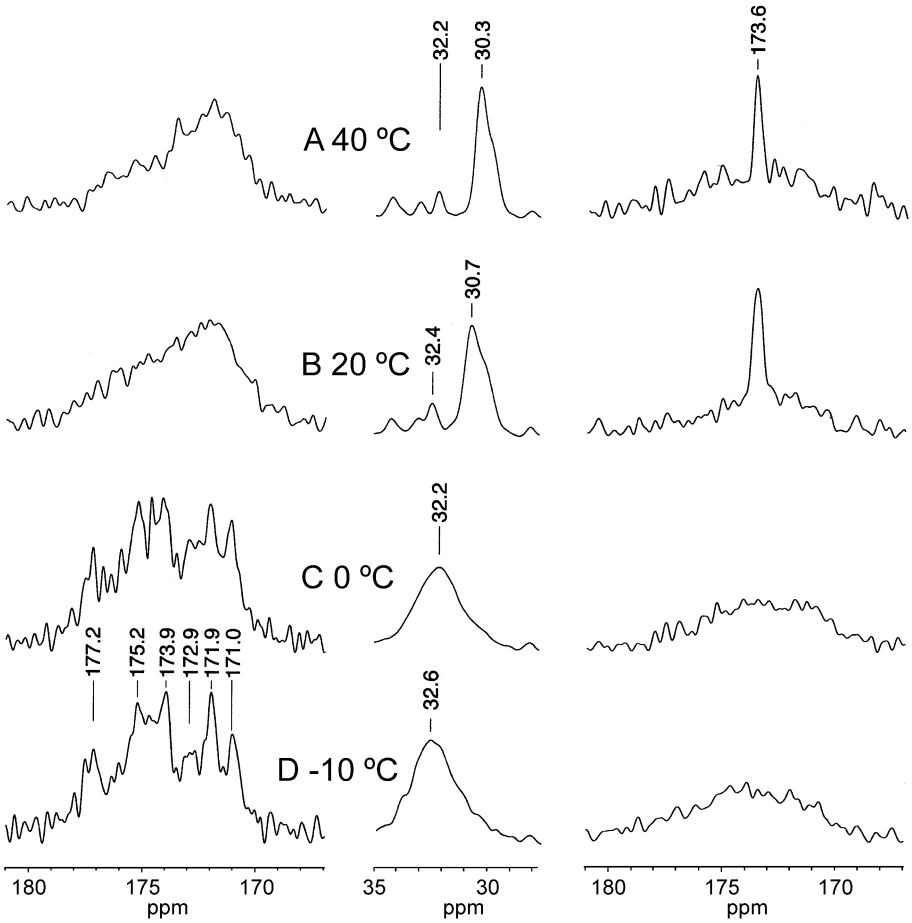

Fig. 5. ^{13}C CP-MAS (left and middle) and DD-MAS (right) NMR spectra of [1-^{13}C]Val-labeled bR reconstituted in DMPC bilayer (1:50 mol ratio) at various temperatures from 40 °C (A) to −10 °C (D).

bilayer at ambient temperature, are recovered at temperatures below 0 °C. The 14 ^{13}C NMR peaks of [3-^{13}C]Ala-bR are thus resolved at −10 °C, instead of the 12 peaks from native PM [32,33]. It is more pronounced in the DD-MAS NMR spectra than the CP-MAS NMR that [3-^{13}C]Ala-labeled NMR peak of the C-terminal α-helix (helix G′) resonated at 15.6 ppm (Fig. 4C, 0 °C) is displaced downfield by 0.4–16.0 ppm at an elevated temperature (Fig. 4A, 40 °C), consistent with the similar temperature-dependent ^{13}C NMR spectra of bR from PM [34]. The lowermost peak of [3-^{13}C]Ala-bR from PM at 17.8 ppm (Ala 196 from the F–G loop) is still suppressed in these reconstituted systems at ambient temperature but is made visible at −10 °C for bR in the DMPC system (Fig. 4D), as a result of taking 2D array [19–25] to be described later. The three uppermost peaks of the reconstituted system, at 14.5, 14.8 and 15.0 ppm, ascribable to Ala residues located near at the vicinity of the ionone ring of retinal, are resonated slightly at higher field positions because of the modified retinal–protein interaction. Naturally, the two intense peaks at 19.7 and 14.1 ppm in the DMPC bilayer are also seen as in the case of egg PC bilayer system (Fig. 2).

As illustrated in Fig. 5, the abovementioned spectral changes could also be monitored by examination of the ^{13}C NMR spectra of [1-^{13}C]Val-labeled bR (left traces), together with the methylene peak-position of fatty acyl chains (middle traces) which is sensitive to lipid phase, either gel (*trans*; 32.6 ppm) or liquid crystalline phase (*gauche*/*trans*; 30.3 ppm) [48]. It should be noted that, in liquid crystalline phase at temperature above 20 °C, the ^{13}C NMR signals of [1-^{13}C]Val-bR exhibit very broad featureless peaks in spite of their peak-positions mainly at the α-helix, whereas the well-resolved ^{13}C NMR peaks similar to those of PM are visible in the gel phase lipids. Closer examination of its CP-MAS NMR spectra at gel phase (C and D), however, shows that the observed spectral pattern is not exactly the same as that of PM: relative proportion of the transmembrane α-helices, 174.5 and 175.5 ppm (Fig. 5C), is not always the same as that of PM (Fig. 3C). Such spectral change was also noted for a variety of mutants in which Schiff base–protein interaction was modified as encountered for D85N [49] and E194Q/E204Q mutant (Saitô et al., submitted), etc. and also bacterioopsin (bO) in which retinal was removed [50].

In Fig. 6, we have illustrated the ^{13}C CP-MAS NMR spectra of [1-^{13}C]Val-(A) and [3-^{13}C]Ala-bR (B) reconstituted into DPPC bilayer at temperatures of 20 and 0 °C (C and D), respectively. Apparently, the ^{13}C NMR pattern at both temperatures are very similar to those reconstituted in the DMPC bilayer (Figs. 4–6). Nevertheless, it should be pointed out that the secondary structure of bR achieved as a 2D array of the reconstituted preparations at low temperature [19,20] is not completely the same as that of the 2D crystal in the PM: the characteristic peak in the ^{13}C NMR signal of [3-^{13}C]Ala 196 from the E–F loop is at 17.8 ppm in PM, but this peak is absent in the reconstituted system. In addition, the relative peak-intensity of the ^{13}C NMR signals

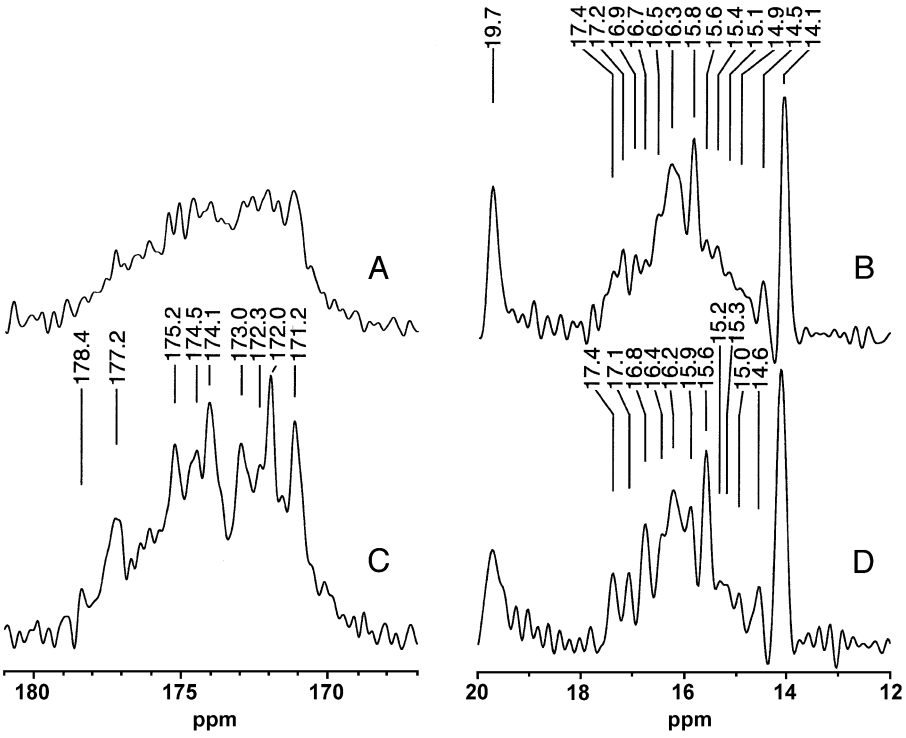

Fig. 6. ^{13}C CP-MAS NMR spectra of [1-^{13}C]Val-labeled bR reconstituted in DPPC bilayer (1:50 mol ratio) at 20 °C (A and B) 0 °C (C and D). ^{13}C NMR signals from carbonyl and methyl regions are given at lower field (A and C) and upper field (B and D), respectively.

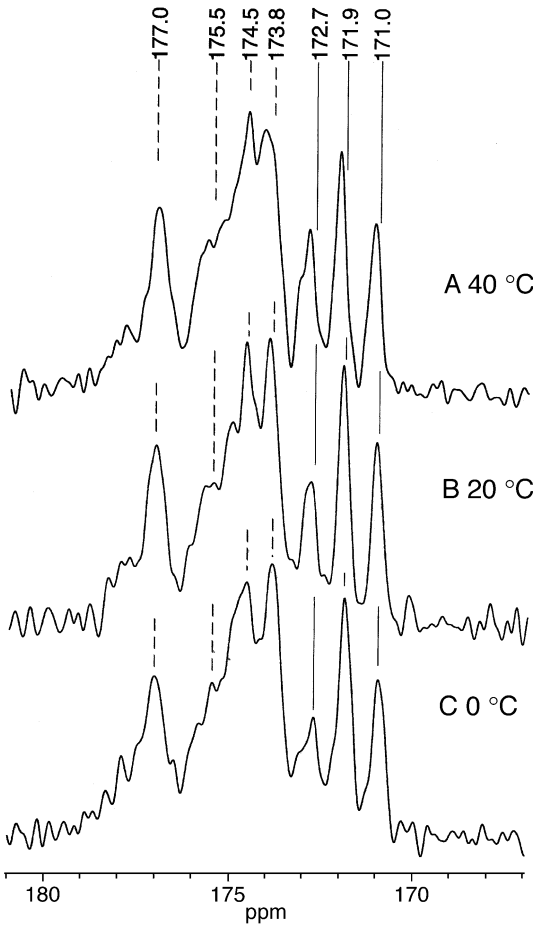

Fig. 7. ^{13}C CP-MAS NMR spectra of [1-^{13}C]Val-labeled bR from PM at various temperatures from 40 °C (A) to 0 °C (C).

of the transmembrane α-helical peaks from [1-^{13}C]Val-bR at 174.6 ppm (including Val 136, 179 and 187 from Table 1; see Fig. 1) is higher than that of 175.0 ppm (including Val 217) in PM, but they are comparable to those of the reconstituted system.

In contrast, it is emphasized that no appreciable temperature-dependent spectral change as mentioned above (Figs. 3–6) is present in PM as illustrated in Fig. 7. It appears that the three signals resonated at the upper field of the loop region (171.0, 171.9 and 172.7 ppm, see Table 1) were displaced downfield when temperature was raised from 20 to 40 °C, leaving the rest of ^{13}C NMR signals unchanged (Fig. 7).

4. Discussion

4.1. ^{13}C NMR spectral feature of monomeric bR

We have demonstrated that ^{13}C NMR signals of [3-^{13}C]Ala-bR of wild-type, arising from PM in the 2D crystalline lattice consisting of the trimeric form, are fully visible at ambient temperature [43]. It turned out, however, that the corresponding [1-^{13}C] and [2-^{13}C]Ala-labeled ^{13}C NMR signals from the loops and transmembrane α-helical regions of bR are almost completely or partially suppressed, respectively [44]. In general, it should be borne in mind that the ^{13}C NMR signals of any given proteins, including collagen fibrils [51] or crystalline peptides [52], are not always fully visible at ambient temperature by CP-MAS NMR, unless otherwise they are completely *static*, free from any kind of backbone and side-chain motions. Naturally, the possibility of internal fluctuations with a variety of motional frequencies should always be taken into account when ^{13}C NMR spectra of fully hydrated proteins are recorded, especially at ambient temperature. As a result, such suppressed ^{13}C NMR signals could be encountered by a failure of attempted peak-narrowing process due to interference of incoherent fluctuations frequency in the order of 10^4–10^5 Hz with coherent frequency of either magic angle spinning or proton decoupling [44–46] when spectra were recorded at 9 T with magic angle spinning of 4 kHz. As a result, it is emphasized that ^{13}C NMR signals from [3-^{13}C]Ala-bR are sensitive to the fluctuation frequency of membrane proteins in the order of 10^5 Hz, while those of [1-^{13}C]Ala- or other amino acid residues labeled bR are sensitive to frequencies of 10^4 Hz. Such a differential response to the fluctuation frequency can be utilized as a very convenient means to distinguish frequency of fluctuation motions present in membrane proteins, on the basis of the ^{13}C NMR studies of the abovementioned two types of ^{13}C labeled proteins [32,33]. In particular, we found that ^{13}C NMR signals from the loop regions of [3-^{13}C]Ala-labeled bR taking blue membranes were completely suppressed either when surface-bound metal ions are completely removed or surface pH is lowered to pH 1.2 [36,53]. It is also found that a considerable proportion of the ^{13}C NMR signals are suppressed from [3-^{13}C]Ala-labeled proteins, especially from the α-helical and loop regions, when its crystalline lattice is disorganized as in bO formed by the removal of retinal from bR [50] or D85N mutant when retinal–protein interaction was modified [49].

It was also shown that single amino acid substitutions in the transmembrane α-helix D of bR such as W80L or W12L are sufficient to disrupt the PM crystalline lattice to result in monomer by specific modification of helix–helix interface [24,25]. Naturally, it is not unexpected that backbone dynamics of resulting monomeric bR is significantly altered from that of bR from PM in the presence or absence of helix–helix and helix–lipid interactions participated in stabilization of the 2D crystalline lattice [24,25]. In fact, we have previously demonstrated that very broad and less intense ^{13}C NMR spectra were observed for [3-^{13}C]Ala-W80L and W12L mutants in which the trimeric structural unit leading to the lattice formation is either disrupted or disorganized due to introduced perturbations to the lipid–helix and helix–helix interactions by mutation of specific residues essential for such interactions [35]. In particular, the ^{13}C NMR peaks from [1-^{13}C]Val- or Ala-labeled these

mutants were almost completely suppressed due to acquisition of motional frequencies interfered with frequency of magic angle spinning [35]. Consistent with these findings, bR integrated into egg PC, DMPC or DPPC bilayer seems to take a monomeric form at ambient temperatures in view of the intense ^{13}C NMR signals at 19.7 ppm from the endogenous lipids from *Halobacteria*, and also broadened and less intense ^{13}C NMR signals (Figs. 2, 4 and 6), similar to those of W80L and W12L mutants [35]. These findings indicate that transmembrane α-helices of monomeric bR and mutants undergo fluctuation motions with frequencies in the order of 10^4 Hz, as compared with these frequencies of PM in the order of 10^2 Hz [44]. In fact, it was shown that bR in the hexagonal lattice is stabilized by at least 5 kcal/mol due to trimer formation [54].

Closer examination of both systems, however, indicates that there are some differences in their respective ^{13}C NMR spectral features: rather well-resolved ^{13}C NMR signals are visible from monomeric [3-^{13}C]Ala-bR integrated into egg PC bilayer as compared with those of W80L and W12L [35], although the ^{13}C NMR peaks from the loops and some transmembrane α-helical regions are still suppressed for both systems. This finding is also consistent with our recent ^{13}C NMR observation of monomeric [3-^{13}C]Ala and [1-^{13}C]Val-labeled phoborhodopsin (*p*pR) reconstituted into egg PC bilayer with or without the transducer [39]. Obviously, such distinct manner of spectral changes between these two systems may be ascribed to the different manner of lipid–protein interactions arising from charged or neutral lipids. It is noteworthy that monomeric [3-^{13}C]Ala-bR mutants grown from *Halobacteria* contains charged endogenous lipids which gave rise to more broadened ^{13}C NMR signals because of strong interaction between bR and such lipids. Therefore, it appears that substitution of the charged endogenous lipids with neutral ones such as egg PC, DMPC and DPPC may result in weakened lipid–protein interactions as a favorable condition to shift the correlation times of fluctuation motions to be able to escape from the region of 10^4 Hz responsible for complete suppression of ^{13}C NMR study on [1-^{13}C]Val-labeled peaks. This may be the reason why the use of egg PC bilayer for bR and *p*pR is more favorable as membrane lipids rather than intrinsic lipids from *Halobacteria* for ^{13}C NMR observations, unless otherwise, oligomerized forms, such as trimeric structures are not readily formed in the native lipid system.

4.2. ^{13}C NMR spectra of bR in 2D array at low temperature

Sternberg et al. [21] showed that bR, purified entirely free of PM lipids and reconstituted into DMPC bilayer, does not arrange into 2D arrays. In fact, it was shown that some specific polar lipids such as phosphatidylglycerol phosphate or phosphatidylglycerol sulfate having a stronger attractive interaction with the protein are required for 2D hexagonal array formation, in the presence of high salt condition, >2 M NaCl, at temperatures below gel to liquid crystalline phase transition [19,20,22,23]. On the basis of such experimental findings on bR, a general model has been proposed for lipid-mediated 2D array formation of membrane proteins in lipid bilayers, on the basis of Monte Carlo simulation [23]. This model arises from a complex consisting of two different lipid species, "annular" or polar lipids and "neutral" lipids and one protein species [23]. Naturally, the presence of the annular lipids in the present bR preparations are characterized by the presence of a peak resonated at 19.7 ppm. The presence of this kind of lipid in bR preparation used here is in favor of the formation of a 2D array at lower temperatures once reconstituted in a neutral lipid bilayer, although a NaCl concentration as low as 10 mM for the sake of convenience for ^{13}C NMR observation still seems to be low as compared with the proposed data of >2 M NaCl [19,20,22,23]. The achieved 2D array at temperature 0 or − 10 °C for DPPC or DMPC bilayers is not always the same as that of PM observed at ambient temperature, because fluctuation motions still remain even at 0 °C at least in the F–G loops as viewed from the suppressed ^{13}C NMR peak of Ala 196 in [3-^{13}C]Ala-bR (Figs. 5C and 6D), as compared with the data of PM at ambient temperature.

In contrast, it appears that naturally occurring 2D hexagonal lattice in native PM is very stable because of the lack of significant temperature-dependent spectral changes for the ^{13}C NMR spectra of [1-^{13}C]Val-labeled bR (Fig. 7). Nevertheless, it should also be taken into account that there appear significant spectral changes in the ^{13}C NMR spectra of [3-^{13}C]Ala-bR, although such changes are mainly restricted to the surface residues, including the surface complex consisting of interhelical loops and C-terminal α-helix protruded from the membrane surface [32,44]. As pointed out previously [31], the hexagonal lattice regulates the dynamics aspect of individual helices, restraining molecular fluctuation of the transmembrane α-helices from 10^4 Hz of monomeric species to 10^2 Hz in the crystalline lattice consisting of the trimeric structures. In this connection, it is also interesting to note that the presence or absence of surrounding charged lipids also play a very important role in determining the dynamic aspect of membrane proteins reconstituted in lipid bilayers. Thus, it is emphasized here that a suitable choice of lipid molecules, as well as appropriate amino acid residues as ^{13}C label [31,33], is also a very important consideration prior to initiating ^{13}C NMR studies on membrane proteins.

5. Concluding remarks

It is found that that well-resolved ^{13}C NMR spectra were recorded for [3-^{13}C]Ala-labeled bR reconstituted into lipid bilayers of egg PC, DMPC or DPPC at *ambient* temperature, although several peaks from the loops and some transmembrane α-helical regions were still suppressed, as compared with those of PM. In addition, it is noteworthy that the observed spectral features for [3-^{13}C]Ala- and

[1-^{13}C]Val-bR surrounded by such neutral lipids turned out to be much better than those of bR mutants surrounded by charged lipids from *Halobacteria* such as W80L and W12L previously reported. This means that backbone dynamics, which is one of the essential determinants for ^{13}C NMR observation in the presence of neutral lipids, is more favorable than that of charged lipids due to modified lipid–protein interaction. Further, we found that ^{13}C NMR signals of PM are almost fully recovered in gel phase lipids of reconstituted DMPC or DPPC bilayers at around 0 °C, although they are not always completely the same as those of PM at ambient temperature. It is therefore concluded that a suitable choice of lipid system, as well as the manner of protein aggregation, is very important to study the conformation and dynamics of membrane proteins by ^{13}C NMR approach.

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