Evidence of local conformational fluctuations and changes in bacteriorhodopsin, dependent on lipids, detergents and trimeric structure, as studied by ^{13}C NMR

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Abstract

We examined how the local conformation and dynamics of [3-^{13}C]Ala-labeled bacteriorhodopsin (bR) are altered as viewed from ^{13}C NMR spectra when the natural membrane lipids are partly or completely replaced with detergents. It turned out that the major conformational features of bR, the α_{II}-helices, are generally unchanged in the delipidated or solubilized preparations. Upon partial delipidation or detergent solubilization, however, a significant conformational change occurs, ascribed to local conversion of $\alpha_{II} \rightarrow \alpha_I$-helix (one Ala residue involved), evident from the upfield displacement of the transmembrane helical peak from 16.4 ppm to 14.5 ppm, conformational change (one or two Ala residues) within α_{II}-helices from 16.4 to 16.0 ppm, and acquired flexibility in the loop region (especially at the F–G loop) as manifested from suppressed peak-intensities in cross-polarization magic angle spinning (CP-MAS) NMR spectra. On the other hand, formation of monomers as solubilized by Triton X-100, Triton N-101 and *n*-dodecylmaltoside is characterized by the presence of a peak at 15.5 ppm and a shifted absorption maximum (550 nm). The size of micelles under the first two conditions was small enough to yield ^{13}C NMR signals observable by a solution NMR spectrometer, although ^{13}C CP-MAS NMR signals were also visible from a fraction of large-sized micelles. We found that the 16.9 ppm peak (three Ala residues involved), visible by CP-MAS NMR, was displaced upfield when Schiff base was removed by solubilization with sodium dodecyl sulfate, consistent with our previous finding of bleaching to yield bacterioopsin. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Lipid–protein interaction plays a crucial role in the physiological activities of integral membrane proteins. bR from *Halobacterium salinarium*, which functions as a proton pump, provides a tractable

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system for exploring the effects of perturbing the lipids on the function of an integral membrane protein [1,2]. In fact, the importance of lipids in the purple membrane (PM) is evident from profound changes in the photochemical cycle kinetics [3,4], the maintenance of native structure over a wide pH range [5], the effect of surface pH [6] and the lipid-dependent conformational changes [7]. The native lipids contain negatively charged polar head groups and highly branched fatty ether (dihydrophytanoyl) chains. The total number of bound lipids per protein after various treatments varies substantially, from ten in intact bR [2] to six after delipidation by DOC [8], and 3–4 after 48 h incubation by CHAPS [9]. Therefore, we anticipated that partial or complete replacement of the native lipids with detergents would cause a variety of local conformational changes of protein backbone in bR at the lipid–water or lipid–protein interfaces, dependent on alteration of the surface charge or the fatty acyl chains, respectively.

It is also anticipated that similar but more pronounced conformational change of membrane proteins could be associated with solubilization by a variety of detergents, although this is an essential step in relation to their purification, measurements with solution NMR, and crystallization. For detergents to yield well-resolved solution NMR signals [9–11], it is important to create a system of small homogeneous micelles that leaves the secondary structure unchanged. This criterion is also valuable for analysis of the re-folding process of membrane proteins in general [12–16]. The X-ray structure of bR at 2.5 Å from microcrystals grown in lipidic cubic phases containing glycerol monooleate has been recently published [17,18], after continuous efforts to grow three-dimensional crystals of bR suitable for diffraction studies [19–21]. Understanding how different detergents and lipids affect protein structure, especially at the lipid–water interface, and how the native protein structure is maintained by specific protein–protein interaction is therefore of importance.

In the present paper, we aimed to clarify how conformation and dynamics of the protein backbone are altered, especially at the lipid–water and lipid–protein interfaces, at ambient temperature upon changing the lipid environment by partial delipidation or solubilization in detergents. For this purpose, we utilized the conformation-dependent displacements of ^{13}C chemical shifts of [3-^{13}C]Ala-bR [22–25] which vary as much as 5–6 ppm depending on particular conformations [26–28] as an intrinsic probe.

2. Materials and methods

2.1. Materials

L-[3-^{13}C]Alanine was purchased from CIL, MA and used without purification. *H. salinarium* S9 and A184G were grown in TS medium of Onishi et al. [29], in which an unlabeled L-alanine was replaced by [3-^{13}C]alanine. Purple membranes from these sources were isolated by the method of Oesterhelt and Stoeckenius [1]. Proteolyis of C-terminus moiety of bR (wild type) was carried out with papain (EC 3.4.22.2) by the method of Liao and Khorana [30].

Delipidation of bR by CHAPS (Sigma, USA) and DM (Dojindo, Japan) and Bio-Beads (BioRad) treatment of PM were performed as described by Seigneuret et al. [9] in the presence of 0.025% (w/v) NaN$_3$. Delipidation by DOC was performed as described by Grigorieff et al. [8]. The delipidated PM thus obtained was washed twice with 20 mM MES, 0.025% (w/v) NaN$_3$ at pH 5. CHAPS/DM treated and Bio-Beads untreated PM was washed twice with 0.4 mM DM, 20 mM MES, 0.025% (w/v) NaN$_3$ at pH 5 to decrease ionic strength. Solubilization of bR by TX-100 and DM was performed as described by Seigneuret et al. [9] with 0.025%(w/v) NaN$_3$. Solubilization by TN-101 was performed under the condition of 5% (w/v) TN-101, 20 mM MES, 0.025% (w/v) NaN$_3$ (pH 5) for 24 h at ambient temperature. Lipid phosphorus was determined by Bartlett assay method.

bR was solubilized also by SDS under the conditions of 2 mg/ml bR, 0.2% (w/v) SDS, 0.025% (w/v) NaN$_3$, 20 mM MES at pH 5, and ambient temperature for 12 h. After incubation, each sample was centrifuged at $39\,800 \times g$ for 1.5 h to remove insoluble fraction. The pH of the solubilized bR was adjusted to pH 5, when necessary, and the resulting solubilized bR was concentrated by ultrafiltration using Centriflo CF 25 (Amicon). Bacterioop-

sin was prepared by bleaching with panchromatic light for 20 h in the presence of 0.5 M hydroxylamine [23]. The pelleted or concentrated bR preparations were placed into a 7.5-mm or 5-mm o.d. zirconia pencil-type rotor. The caps were tightly glued to rotor by rapid Alardyte to prevent leakage of water from the samples during magic angle spinning for NMR measurements. Absorption spectra of these preparations were measured on a Shimadzu UV 2000 UV-visible spectrophotometer.

2.2. NMR measurements

100.7 MHz high-resolution ^{13}C NMR spectra were recorded in the dark at 20°C on a Chemagnetics CMX-400 NMR spectrometer either by cross-polarization magic angle spinning (CP-MAS) or dipolar decoupled magic angle spinning (DD-MAS) with single pulse excitation. The spectral width, contact and acquisition times for the former experiment were

40 kHz, 1 ms and 25 ms, respectively. Repetition time was 4 s for both the CP and DD experiments. The $\pi/2$ pulses for carbon and proton were 5–6 µs and 4.5–5 µs for 7.5 and 5 mm probes, respectively, and the spinning rates were 2.6 kHz. Free induction decays were acquired with data points of 1 K and Fourier transforms were carried out as 8 K points after 7 K points were zero-filled. ^{13}C chemical shifts were first referred to the carboxyl signal of glycine (176.03 ppm from TMS) and then expressed as relative shifts from the value of TMS.

Spectral deconvolution was performed using a software written by H. Kurosu and I. Ando for a NEC personal computer or a commercial package, PeakFit for Windows by SPSS, IL, USA. The peak-fitting procedure was repeated until the difference between the experimental and calculated spectra is minimized by inspection after feeding the experimentally resolved peak positions only and appropriate gaussian linewidths (halfwidths of 0.2–0.4 ppm).
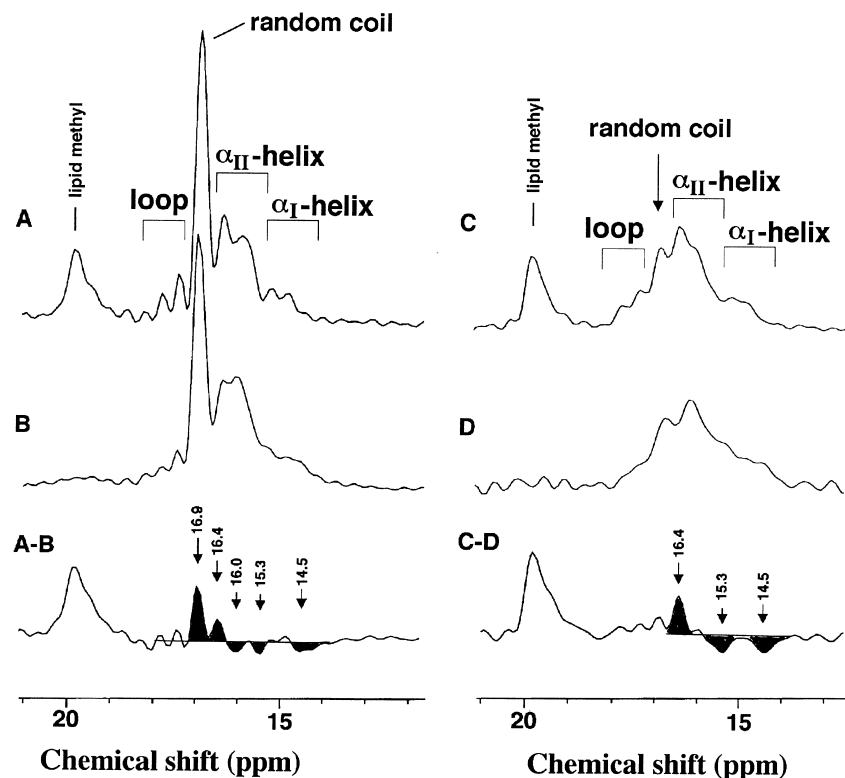

Fig. 1. 100.7 MHz ^{13}C DD-MAS NMR spectra of [3-^{13}C]Ala-labeled intact (20 mM MES, 0.025(w/v) NaN$_3$, pH 5) (A) and papain-cleaved (C) bR and CHAPS/DM-treated preparation of intact bR (B) and papain-cleaved preparation (D), and their difference spectra (A,B and C,D).

3. Results

Fig. 1 shows ^{13}C DD-MAS NMR spectra for [3-^{13}C]Ala-labeled intact and papain-cleaved bR at pH 5 (Fig. 1A and C, respectively) and the corresponding CHAPS/DM-treated preparations (Fig. 1B and D, respectively), and their difference spectra. It is noteworthy that two signals from three Ala residues (at 16.9 and 16.4 ppm) are displaced upfield by the partial delipidation. As expected, the signal of ^{13}C-labeled lipid methyl groups (19.9 ppm), transferred from [3-^{13}C]Ala residues [24], was completely removed in these delipidated preparations. Region-specific assignment of these peaks to bR is based on the conformation-dependent displacement of peaks, as described in our previous papers [22,23], except for the peak designated by the arrow in trace C, to be discussed later. Similar displacement of peaks, without the peaks at 16.9 and 16.0 ppm, is noted for the difference spectrum of the papain-cleaved preparation. Fig. 2 illustrates the ^{13}C CP-MAS NMR spectra of [3-^{13}C]Ala-bR partially delipidated by a variety of detergents, DOC, CHAPS and CHAPS/DM (Fig. 2B–D), together with the spectrum of intact bR (Fig. 2A) and the deconvoluted spectra demonstrated at the right-hand side. The 12 well-resolved peaks, which were very recently observed for PM at pH 7 (unpublished data), and an additional single peak at 14.5 ppm were used as individual components for the deconvoluted spectra for PM and its partially delipidated preparation at pH 5. In practice, however, the four peaks, 17.4, 16.9, 16.0 and 14.9, were treated as single peaks here. The estimated total intensities of the peaks
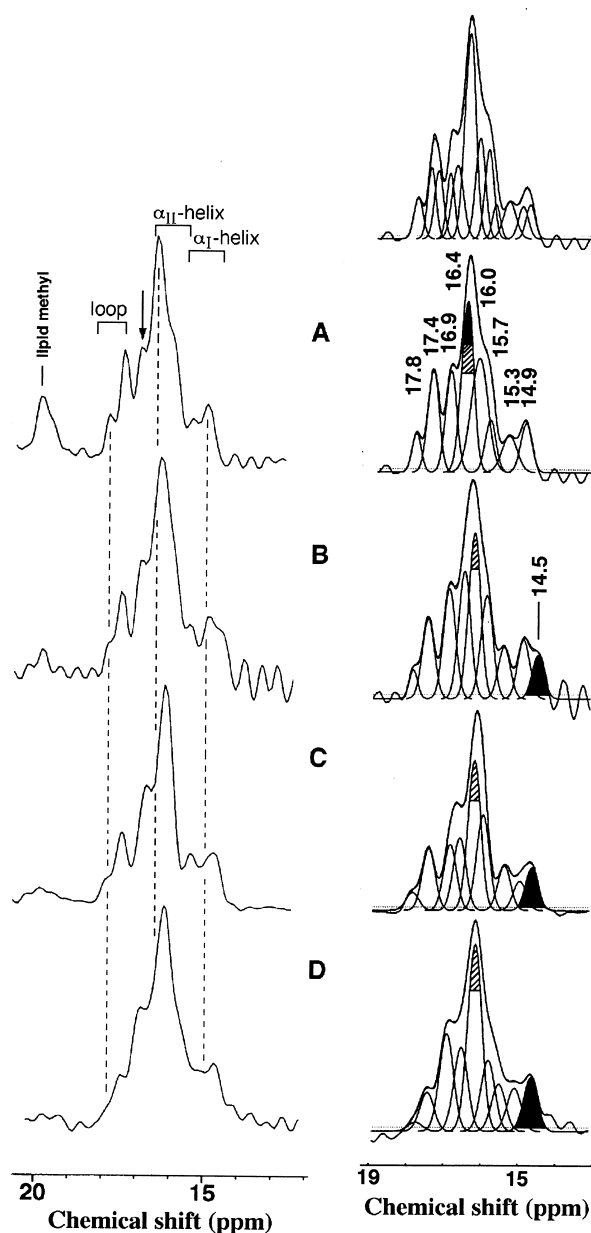
Table 1
Absorption maximum (nm) of bR in the presence of a variety of detergent molecules

	Detergents used for delipidation or solubilization	Intact	Delipidated	Solubilized
bR	DOC	568	562	
	CHAPS		560	
	CHAPS/DM		558	
	DM			550
	TX-100			550
	TN-101			550
	SDS			437

Fig. 2. 100.7 MHz ^{13}C CP-MAS NMR spectra of [3-^{13}C]Ala-bR in PM (20 mM MES, 0.025% (w/v) NaN$_3$, pH 5). Intact pellet (A), DOC-treated (B), CHAPS-treated (C) and CHAPS/DM-treated PM (D). Corresponding deconvoluted spectra based on the experimentally resolved 12 (top) or 8 or 9 peaks are illustrated on the right-hand side. The peaks whose intensities are changed by treatment with these detergents are painted out with black and hatched peaks. For the assignment of the peak denoted with arrow, see text.

for the CP-MAS NMR spectrum of intact bR are 22 ± 1 by assuming the peak-intensity for the resolved peak at 17.8 ppm (Ala 196 on the basis of
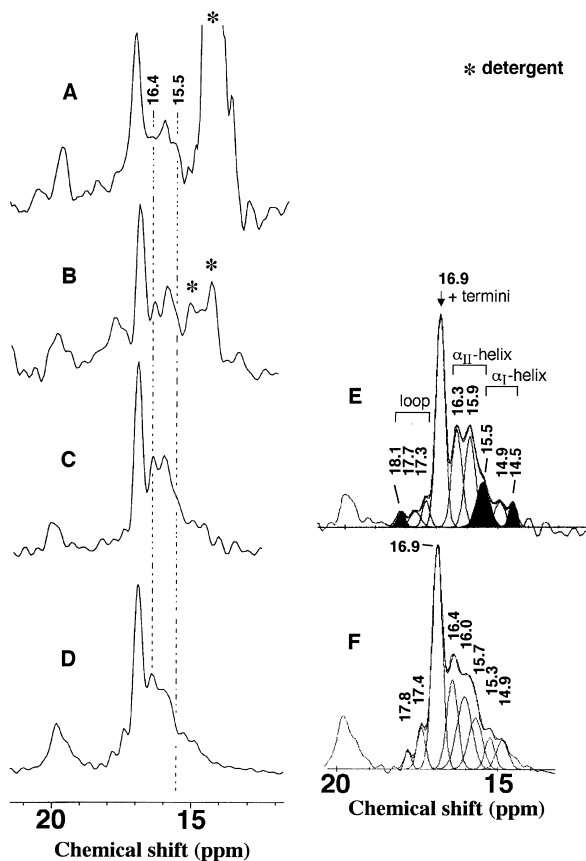
Fig. 3. 100.7 MHz ^{13}C DD-MAS NMR spectra of [3-^{13}C]Ala-bR solubilized in DM micelle (A), solubilized in TN-101 micelle (B), solubilized in TX-100 micelle (C), and intact PM (D). The spectra E and F on the right panel are obtained by the deconvolution of spectra C and D, respectively. The peaks whose intensities are changed by solubilization are painted out with black.

mutant spectra; to be published) as unity, in fairly good agreement with the 22Ala residues in the protein other than N- or C-terminus. Distinct spectral changes (from 16.4 to 14.5 ppm (blacked peaks) and from 16.4 to 16.0 ppm (hatched peaks)) appear upon

these treatments as manifested from the peak-intensities painted out with black, even though the absorption maxima of the chromophore in the visible spectra were almost the same (Table 1). It is notable, however, that no pronounced enhancement of the peak at 15.3 ppm is evident in the CP-MAS NMR (Fig. 2), in spite of the presence of the enhanced peak in the DD-MAS NMR (Fig. 1). Such suppression of signals in the CP-MAS NMR spectra occurs at residues undergoing rapid fluctuation. In particular, we found that ^{13}C NMR signals of at least one Ala residue is missing from the CP-MAS NMR spectrum of intact bR, when it was carefully compared with the corresponding DD-MAS NMR spectrum or CP-MAS NMR spectrum of mutant such as A184G (manuscript in preparation). The peak at 14.5 ppm is present in both types of spectra. In addition, it is interesting to note that the relative peak-intensities of the ^{13}C NMR signals from the loop region, between 17.8 and 17.4 ppm, are decreased in the DOC-, CHAPS- and CHAPS/DM-treated preparations as compared with intact bR due to reduced CP efficiency arising from the acquired flexibility, although no displacements of peaks are seen in the helical region, except for the peak at 14.5 ppm. It is also mentioned that no such suppression of peaks was notable in the DD-MAS NMR spectra shown in the case of intact and CHAPS/DM-treated preparations (Fig. 1).

The absorption maxima of the solubilized bR preparations were all about 550 nm after the treatment with DM, TX-100 or TN-101 as summarized in Table 1, i.e., appreciably blue-shifted from that of intact bR (568 nm). These solubilized bR preparations seemed to be monomeric because no sedimentation was evident upon centrifugation at $202\,000 \times g$. We recorded the ^{13}C DD-MAS NMR spectra of

Table 2
The presence of α_{II}-helices as major components as characterized by NMR and IR measurements

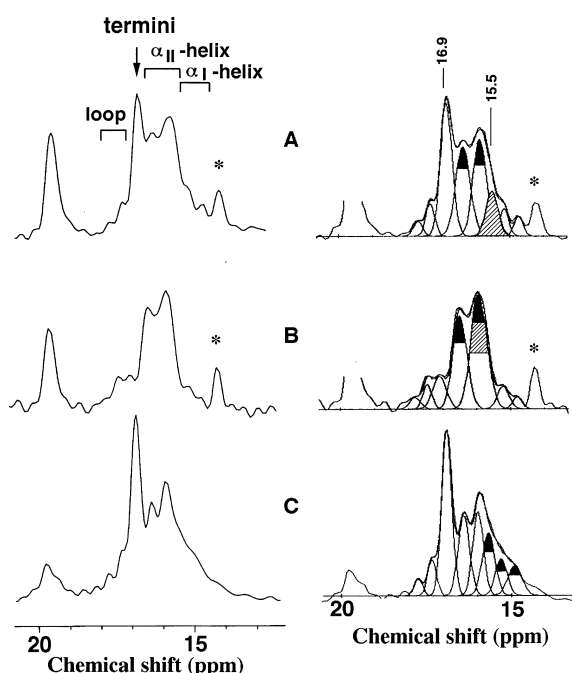|	NMR	Ref.	IR	Ref.
PM	Yes	[22]	Yes	[37]
Deionized blue membrane	Yes	Unpublished	Decreased α_{II} content	[38]
bO	Yes	[23]	Yes	[37]
bR fragments in lipid bilayer	Yes	[39]		
Delipidated bR with TX-100			Increased α_I character	[7]
Delipidated bR with DOC, CHAPS, DM	Yes	This work		
Solubilized bR (with TX-100 or SDS)	Yes	This work	No	[37]

Fig. 4. 100.7 MHz ^{13}C DD-MAS NMR spectra (pH 5) of [3-^{13}C]Ala-bR dissolved in SDS micelle: bR (A) and its papain-treated preparation (B) together with ^{13}C NMR spectrum of bO (pH 7) (C). The increased peak-intensities with extent of the peak at 16.9 ppm are painted out with black. Hatched peaks (15.5 and 15.9 ppm for the A and B, respectively) are ascribed to the peaks whose intensities are changed by solubilization. Asterisk-labeled peaks arose from the detergent used.

[3-^{13}C]Ala-bR, solubilized in TX-100, TN-101 and DM (Fig. 3). The overall spectral features of these preparations are very similar, although some signals that were resonated at fields higher than 15.5 ppm overlapped with the asterisk-labeled signals from the detergents in the cases of TN-101 and DM. In Fig. 3E, the ^{13}C DD-MAS NMR of [3-^{13}C]Ala-bR solubilized in TX-100 is deconvoluted into nine components without overlap with ^{13}C NMR signals of the detergent. It is noteworthy that the presence of additional three peaks, 18.1, 15.5 and 14.5 ppm, as compared with the spectrum of the intact PM (Fig. 3F) is characteristic of the monomeric form in this sample. Surprisingly, solubilized bR both in TX-100 and N-101 gave rise to the ^{13}C CP-MAS NMR spectra, although their peak-intensities are appreciably decreased when they are compared with those of intact PM (spectra not shown).

The ^{13}C DD-MAS NMR spectra are visible for SDS-solubilized bR (Fig. 4A: see Table 1) and its

papain-cleaved preparation (pbR) (Fig. 4B) and bO (pH 7) (Fig. 4C). In addition to the presence of the peak at 15.5 ppm (the hatched peak), as observed in the cases of solubilization of TX-100 (Fig. 3), the relative proportion of the α_{II}-helical peaks (16.4 and 15.9 ppm) in [3-^{13}C]Ala-bR is appreciably increased (painted out with black; three to four Ala residues) at the expense of the peak-intensity of 16.9 ppm resonating at the peak-position corresponding with the random coil peak (eight Ala residues remained; see the deconvoluted spectra at the panel of the right-hand side). Obviously, the conformational change as manifested from the displacement of peaks from 16.9 ppm to 16.4–15.5 ppm (blacked) induced by SDS arose from regions other than the C-terminus, because the peak-intensities at 16.4–15.5 ppm remain unchanged with and without treatment with papain (Fig. 4A,B). The hatched peak is displaced downfield from 15.5 to 15.9 ppm in papain-cleaved preparations, due to a probable local conformational change as a result of cleavage of the C-terminus, as encountered previously [31]. Only the two Ala residues for SDS-solubilized bR remained to contribute to the peak at 16.9 ppm. It is interesting to note that no enhanced peak appears at 15.5 ppm in bO, and the resulting enhanced peaks (painted out with black) are not the same as those of SDS-treated samples. No information is available
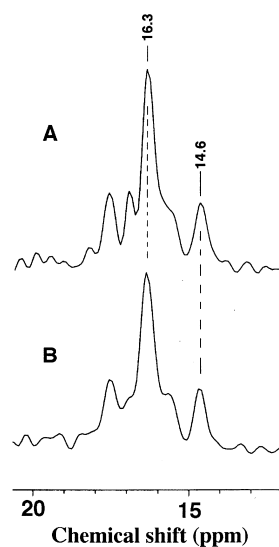

Fig. 5. 100.7 MHz ^{13}C CP-MAS NMR spectra of CHAPS/DM-treated BioBeads-untreated [3-^{13}C]Ala-bR (A) and its papain-treated preparation (B) (20 mM MES, 0.025% (w/v) NaN$_3$, pH 5).

whether the peak at 14.5 ppm, characteristic of the delipidation and solubilization, is visible in SDS-solubilized sample, because this peak may overlap with the signal from SDS. In Fig. 5, we show that the peak-position of the displaced major transmembrane helical peak is recovered from 16.1 to 16.3 ppm in the presence of DM for CHAPS-treated PM (A) and its papain-treated preparation (B), although the peak at 14.6 ppm remains when the natural lipids are replaced with DM. It is worth mentioning here that no signal was available from DM under the conditions of CP-MAS experiment.

4. Discussion

4.1. ^{13}C NMR spectra of delipidated or solubilized bR

The assignment of ^{13}C NMR peaks for bR so far discussed [22–25] can be also extended to delipidated or solubilized bR preparations, because it turned out that the local conformations, including the α_I-, α_{II}-helices and loop structures, are mainly retained. Here, the ^{13}C NMR chemical shifts for the α_I-helix (ordinary helix) and α_{II}-helix are referred to those of $(Ala)_n$ in the solid state [22–24] and in hexafluoro-isopropanol (HFIP) following the proposal by Krimm and Dwivedi [32], respectively. The present finding by NMR is consistent with the data of absorption maxima (562–550 nm) which are not greatly different from the maximum of the intact sample (568 nm), as summarized in Table 1. The relative peak-intensities vary, depending on the type of preparations, and reflect subsequent conformational changes, however. It is known that delipidated preparations with CHAPS and CHAPS/DM have photochemical cycles with changed kinetics [3].

4.2. Specific conformational change of protein backbone due to delipidation

As mentioned already, two kinds of conformational changes are noteworthy for the protein backbone of detergent-treated bR: (1) the $\alpha_{II} \rightarrow \alpha_I$ conversion as manifested from the appreciable displacement of a peak (one Ala residue) from 16.4 to 14.5 ppm, and (2) the conformational change (two Ala residues involved) within the α_{II}-helix (16.4 to 16.0 ppm) by

delipidation. It was previously shown that the structure, viewed as the projection map after DOC treatment, is very close to that of the intact sample [8]. This means that removal of four of the ten lipids per protein does not seriously affect the overall conformation as far as cryo-electron microscopic data are concerned. The present NMR observation indicates, however, that there is a major conformational change for at least one Ala residue, involving an $\alpha_{II} \rightarrow \alpha_I$ conversion, when the strongly bound lipids are either removed (Figs. 1 and 2) or replaced with DM (Fig. 5). This conformational change is induced in the absence of branched fatty acyl chains due to the delipidation, and not recovered completely without the natural lipids (Fig. 5). The displacement of the peak from 16.9 ppm to 16.0 ppm in intact bR and the absence of the corresponding peak change in the papain-cleaved preparation are readily explained by stabilization of the protruding α-helix at the C-terminus due to increased surface pH, as a result of the missing negative charge of the natural lipids and the pH-dependent conformation reported previously [31].

The additional upfield displacement of the most intense transmembrane helical signal by 0.4 ppm in the CP-MAS NMR spectra, within the range of α_{II}-helix, due to the treatment with CHAPS and CHAPS/DM, can be interpreted in terms of a conformational change of protein backbone (for two Ala residues), as defined by either a change of a set of torsion angles of the peptide unit or different manner of conformational fluctuation, discussed in more detail later. The latter conformational change (from 16.4 to 16.0 ppm) could be readily recovered (from 16.1 ppm to 16.3 ppm) when DM was added to the preparation delipidated by CHAPS as substituent for the naturally occurring lipids (Fig. 5). It is also noteworthy that the relative peak-intensities of the loop region, 17.8 and 17.4 ppm, in the CP-MAS NMR spectra (Fig. 2) appreciably decreased when PM was delipidated with CHAPS or CHAPS/DM. This is more evident for the peak 17.8 ppm ascribable to Ala 196 which is located at the F–G loop. The conformation of these regions is unchanged in spite of the decrease in these peak-intensities, as seen in the deconvoluted spectra in Fig. 2. To prove this, there appears no such an intensity change in the DD-MAS NMR (Fig. 1). Such an obvious reduction of the

peak-intensities arises in many instances when the rate of cross-polarization is appreciably decreased due to enhanced flexibility in these loop regions. In native bR, such a conformational flexibility for the loop regions may be controlled mainly by electro-static interactions among cations, negatively charged head groups of lipids, positively or negatively charged side-chains of protein backbone, etc. In fact, we recently showed that either lowering surface pH [31] or deletion of cationic groups [24] caused significant spectral changes, ascribed to a local con-formational change. Moreover, we also noted that conformation of the loop regions is almost com-pletely disordered when metal ions are completely removed, as in deionized blue membrane (Tuzi et al., in preparation). It is worthwhile to point out here that the conformation of the loop region is not recovered when DM is added to CHAPS-treated PM, in spite of the recovery in the transmembrane region (Fig. 5). Accordingly, it is probable that deli-pidation by the detergent removes also metal ions and substantially modifies surface pH, resulting in enhanced flexibility in the looped region.

4.3. Conformational changes due to solubilization

We found that TX-100 solubilized preparation gave rise to a ^{13}C DD-MAS NMR spectrum similar to that of intact bR, but with an additional three peaks at 14.5, 15.5 and 18.1 ppm (Fig. 3). This means that solubilization caused conformational changes of both transmembrane helices (14.5 and 15.5 ppm) and loop (18.1 ppm) through perturbation to respective torsion angles. In contrast to our expectation, the spectral resolution was not always improved by sol-ubilization with this detergent as compared with that of bR pellets. This finding is consistent, however, with the previous ^{13}C NMR study on [1-^{13}C]Phe-la-beled bR by Seigneuret et al. [10], as recorded by ^{13}C CP-MAS NMR spectra. The presence of the above-mentioned enhanced peak at 15.5 ppm seems to be characteristic of the monomeric form of bR as seen in all of the solubilized bR preparations (Fig. 3). It turned out that the peak at 18.1 ppm was reprodu-cible in subsequent measurements and its peak-inten-sity was increased at lower temperature (10°C). It is interesting that the 15.5 ppm peak was also made visible when bR was solubilized by SDS. This is be-cause the native secondary structure of bR is now disrupted by solubilization with this detergent. The most prominent spectral change due to solubilization by SDS is displacement of the peak at 16.9 ppm upfield, consistent with our previous observation for bleaching to yield bO [23]. The resulting confor-mation of bR solubilized in SDS is obviously not the same as that of bR (Fig. 4).

4.4. α_{II}-Helices in delipidated and solubilized preparations

The α_{II}-helices found in PM constitutes the dom-inant conformation in the delipidated and solubilized bR also. The existence of the α_{II}-helices as the major transmembrane structures of bR had been initially proposed by Krimm and Dwivedi [32] to account for an abnormally high amide I frequency of IR spectra, and subsequently confirmed by a number of spectroscopic techniques including IR [33,34], Raman [34], UVCD [35] and far-UV oriented CD [36]. As summarized in Table 2, there appears a dis-crepancy in the assignment of the α_{II}-helical form between the IR and the NMR measurements: the α_{II}-helix as viewed from IR spectra [7,37,38] is com-pletely or substantially converted to the α_{I}-form when bR is partially delipidated, solubilized or de-ionized as blue membrane, but such conversion ap-pears minimal by NMR (only a single Ala residue involved). In particular, the α_{II}-helix disappears for the monomer solubilized by SDS micelles in IR measurements [37], even though the α_{II}-helical con-tent in the ^{13}C NMR spectra of Ala residue is in-creased. It is probable that this kind of discrepancy might arise from differences in time scale between IR and NMR spectra, because conformation and its flexibility are not always separable as independent parameters as far as NMR measurements on mem-brane proteins. In fact, we had found that Ala resi-dues incorporated into the transmembrane helices of synthetic fragments of bR take the α_{I}-form when recorded in the solid state dried from hexafluoroiso-propanol solution, but the same fragments behaved as the α_{II} form when inserted in the lipid bilayer [39]. It is probable that conformational fluctuation of the transmembrane segment in the bilayer behaves as an α_{II}-helix, although further study is necessary to clar-ify this point.

Acknowledgements

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