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Dynamic Structure of Bacteriorhodopsin Revealed by ^{13}C Solid-state NMR

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We demonstrate here a dynamic structure of bacteriorhodopsin (bR) as revealed by ^{13}C NMR studies on [3-^{13}C]-, [1-^{13}C]Ala- and/or Val-labeled wild type and a variety of site-directed mutants at ambient temperature. For this purpose, well-resolved (up to twelve) ^{13}C NMR peaks were assigned with reference to the displacement of peaks due to the conformation-dependent ^{13}C chemical shifts and reduced peak-intensities due to site-directed mutations. Revealed bR structure was not rigid as anticipated from 2D crystals of hexagonal array but a dynamically heterogeneous, undergoing a variety of local fluctuations depending upon specific site with frequency range of $10^2 - 10^8$ Hz. In particular, dynamics-dependent suppression of peaks turned out to be very sensitive to the motion of 10^{-4} s and 10^{-5} s interfered with frequency of magic angle spinning and proton decoupling, respectively. It is also noteworthy that such dynamic feature is strongly dependent upon the manner of 2D crystalline packing: ^{13}C NMR peaks of monomeric bR yielded either highly broadened or completely suppressed signals, depending upon the type of ^{13}C-labeled amino-acid residues.

Key words: bacteriorhodopsin, ^{13}C solid-state NMR, conformation-dependent ^{13}C chemical shifts, 2D crystals, dynamic structure, magic angle spinning, proton-decoupling frequency, site-directed mutation

INTRODUCTION

Bacteriorhodopsin (bR) from *Halobacterium salinurum* is assembled into naturally occurring two-dimensional (2D) crystalline patches known as purple membrane in which its trimeric unit is hexagonally packed under physiological condition. Three-dimensional (3D) structures of bR in the 2D or 3D crystals have been so far determined by either cryo-electron microscope or X-ray diffraction, respectively, at low temperature. Revealed structures of the trans-membrane α-helices are generally consistent but their surface structures are either obscured or inconsistent among the data so far published. One reason for this inconsistency is that they are not always static as anticipated but undergoing motional fluctuations with various frequencies. In addition, their averaged structures could be severely

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modified by a variety of intrinsic or environmental factors such as ionic strength, temperature, pH, site-directed mutation, or crystallographic contacts [1]. To clarify this problem, we have demonstrated that a ^{13}C NMR approach on ^{13}C-labeled proteins proved to be an excellent, non-perturbing means suitable for a study at ambient temperature, if resulting ^{13}C NMR signals were well resolved and site-specifically assigned to amino-acid residues located at specific position [1-3].

ASSIGNMENTS OF ^{13}C CHEMICAL SHIFTS

Isotope enrichment of bR by ^{13}C nuclei through large-scale culture of *H. salinurum* is prerequisite for the present NMR approach, in order to improve both *sensitivity* and *selectivity* of particular amino- acid residues under consideration. In particular, selective isotope labeling, using single specie of replaced ^{13}C-labeled amino acid residues such as [3-^{13}C]-, [1-^{13}C]Ala (circled) or Val (boxed)-labeled amino acids in the synthetic medium, could be most favorable over an alternative approach using uniform labeling technique, in terms of the maximum sensitivity enhancement free from additional broadening due to scalar ^{13}C-^{13}C couplings [4] (see Figure 1 for the primary sequence of bR).

As an illustrative example, Figure 2 illustrates the ^{13}C NMR spectra of [3-^{13}C]Ala-labeled bR at ambient temperature, recorded both by dipolar decoupled magic angle spinning (DD-MAS) (A) and cross polarization-magic angle spinning (CP-MAS) (B), respectively. Twelve ^{13}C

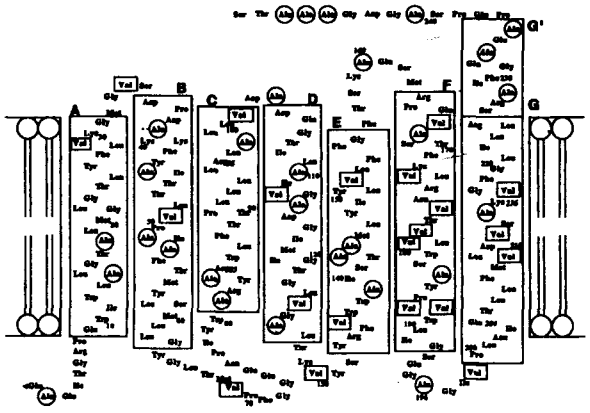

Figure 1 Primary sequence of bR taking into account of secondary folding. Ala and Val residues are circled and boxed, respectively.

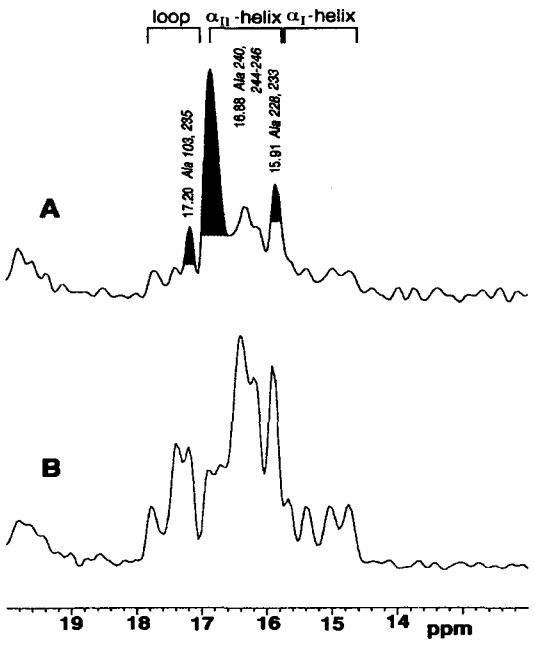

Figure 2 ^{13}C NMR spectra of [3-^{13}C]Ala-labeled bR. (A) DD-MAS and (B) CP-MAS spectra. The ^{13}C NMR signals from the C-terminal residues in the upper trace are in gray [3].

NMR signals, including the five single carbon signals, are well resolved under the condition of excess hydration (pelleted preparation) among 29 Ala residues in bR. The three intense signals marked by gray in the ^{13}C DD-MAS (Figure 2A) are suppressed in the corresponding CP-MAS spectrum (Figure 2B), although the spectral features of the rest are unchanged. This is because ^{13}C NMR signals from flexible C-terminal tail are suppressed in the latter, due to the presence of rapid motions with correlation time in the order of 10^{-8} s. The regiospecific assignment of these peaks to α-helices or loops can be made with reference to the compiled conformation-dependent displacements of C_α, C_β and C=O peaks of reference data [2]. Further site-specific assignment of the peaks can be performed by location of the reduced peak from the site-directed mutant which lacks the signals of amino acid residue of interest as compared with the wild type [1,3]. In this way, more than 60% of the ^{13}C NMR signals has been assigned for [3-^{13}C]Ala-bR and utilized to examine local conformation of specific residues. In particular, we were able to identify the presence of C-terminal α-helix (G' helix in Figure 1) protruding from the membrane surface from the ^{13}C chemical shift data of [3-^{13}C]Ala-labeled residues, although such structure could not be identified by X-ray diffraction methods.

Nevertheless, ^{13}C NMR signals from labeled probes are not always visible when proteins embedded in membranes are undergoing random fluctuation motions of *incoherent* frequency interfered with *coherent* frequency of magic angle spinning (10^4 Hz) or proton decoupling (10^5 Hz), to result in failure of the attempted peak-narrowing [1,3]. In fact, we found that ^{13}C NMR signals from [1-^{13}C]- or [2-^{13}C]Ala- labeled bR of wild type were suppressed when these probe molecules are located at the interhelical loops or interfacial region of the transmembrane α-helices. In addition, ^{13}C NMR signals of multiply labeled proteins such as [1,2,3-$^{13}C_3$]Ala-bR are not useful as probe molecules because of substantial line-broadening [4]. It should be pointed out, however, that ^{13}C NMR signals of [1-^{13}C]Val-labeled bR are normally visible [5].

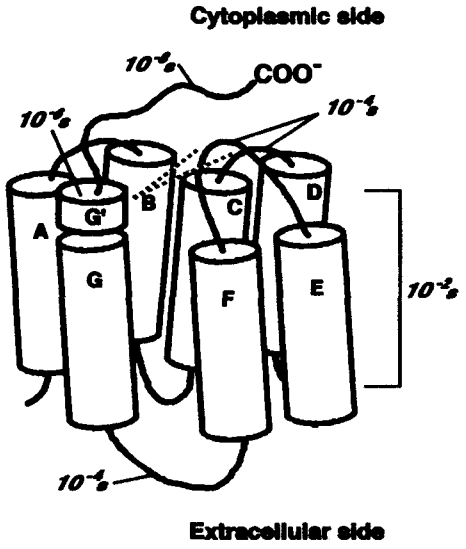

Figure 3 Schematic representation of dynamic structure of bR as revealed by ^{13}C NMR

DYNAMIC PICTURE OF BR

It turned out that bR structure at ambient temperature as revealed by the current ^{13}C NMR data is not rigid as anticipated from X-ray diffraction as obtained at low temperature but dynamically heterogeneous undergoing

motional fluctuation of various ranges of frequencies (or correlation times as reciprocal numbers) as viewed from the dynamics-dependent suppression of peaks as mentioned above, as schematically illustrated for wild type (Figure 3).

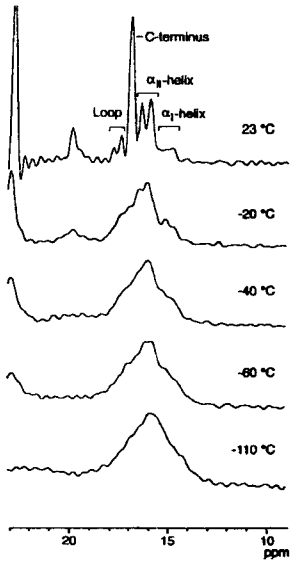

Figure 4 Temperature-dependent change of ^{13}C DD-MAS NMR spectra of [3-^{13}C]Ala-bR [2]

As to the dynamics of the transmembrane α–helices, it is noted that the well-resolved ^{13}C NMR signals of bR observed at ambient temperature are broadened considerably at temperature below -20°C as illustrated in Figure 4. This is the reason why exchange processes with time scale of 10^{-2} s are present in this region at ambient temperature among several slightly different conformations.

Naturally, it is expected that such dynamic picture of wild type could be substantially modified when lipid-protein or retinal-protein interactions were altered as in W80L mutant without hexagonal lattice (submitted) or bacterio-opsin (bO) and D85N mutant at alkaline pH lacking Schiff base [6] or its charge [7], respectively. In the former, it was shown that the individual transmembrane α-helices of W80L mutant acquired accelerated fluctuation motions with time scale of 10^{-4} s, instead of 10^{-2} s in the wild type, to yield very weak or suppressed ^{13}C NMR signals. Such fluctuation motions are obviously caused by the absence of trimeric complex and are unfavorable for ^{13}C NMR experiments, especially when [1-^{13}C]Val probes were utilized. Significant conformational and dynamics changes associated with modified retinal-protein interactions were also noteworthy in the transmembrane α-helices and loops, including B and G helices, B-C, E-F and F-G loops, with correlation times in the order of 10^{-5} s [6,7]. Undoubtedly, capability of such changes in bR is very important from biological point of view, in order to allow entry of retinal at the initial stage of protein folding or to facilitate the efficient proton release and uptake through membrane.

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