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COMMUNICATION

Advances towards resonance assignments for uniformly—^{13}C, ^{15}N enriched bacteriorhodopsin at 18.8 T in purple membranes

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Abstract Solid state NMR spectra from uniformly ^{13}C, ^{15}N enriched bacteriorhodospin (bR) purified from *H. salinarium* were acquired at 18.8 T using magic angle spinning methods. Isolated resonances of 2D $^{13}C-^{13}C$ spectra exhibited 0.50–0.55 ppm line-widths. Several amino acid types could be assigned, and at least 12 out of 15 Ile peaks could be resolved clearly and identified based on their characteristic chemical shifts and connectivities. This study confirms that high resolution solid state NMR spectra can be obtained for a 248 amino acid uniformly labeled membrane protein in its native membrane environment and indicates that site-specific assignments are likely to be feasible with heteronuclear multidimensional spectra.

Keywords Solid state NMR · Bacteriorhodospin · Assignment · Membrane protein

Abbreviations

NMR	Nuclear magnetic resonance
bR	Bacteriorhodospin

Introduction

Here, the feasibility of making resonance assignments of solid state NMR spectra from a uniformly ^{13}C, ^{15}N enriched, large integral membrane protein, bacteriorhodospin (bR) is demonstrated as a first step in structural studies. In solution NMR, sequence-specific resonance assignments are routinely extracted from multidimensional spectra of usually uniformly ^{13}C, ^{15}N, and ^{2}H enriched proteins, although membrane proteins still represent a major challenge due to the large size and concomitant slow tumbling of either protein-detergent micelle complexes or when membrane-embedded. Thus, it is a notable accomplishment that in uniformly enriched (^{2}H, ^{13}C, ^{15}N) micelle-bR complexes the residues were assigned by solution NMR (Schubert et al. 2002), mainly in the mobile loop regions. Solid state NMR offers the advantage of studying large membrane proteins at atomic resolution in a variety of sample forms, including their native membrane environment. However, the assignment of uniformly or extensively labeled proteins has become feasible only recently as a result of important developments in solid state NMR, ranging from sample preparation to hardware, pulse sequence development and high fields. Nonetheless, membrane protein assignment is still very challenging, which is reflected by the fact that only few partial assignments of extensively ^{13}C, ^{15}N enriched integral membrane proteins are available to date (Egorova-Zachernyuk et al. 2001; Etzkorn et al. 2006; Jehle et al. 2006; Kobayashi et al. 2006; Lange et al. 2006a, b; van Gammeren et al. 2005a, b; Varga et al. 2007 b), mostly published in the past 2 years.

Bacteriorhodopsin of *H. salinarium* is a 26 kDa light-driven proton pump of 248 amino acids (per monomer) which folds into 7-transmembrane spanning helices. Bacteriorhodopsin expresses in very high concentration and forms 2D crystals in the purple membranes, which can be readily isolated (Oesterhelt and Stoeckenius 1974). Since its discovery (Oesterhelt et al. 1971, 1973), it has been a

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subject of numerous solid-state NMR studies not only because of its importance, but also its favorable characteristics: it is readily available in milligram quantities suitable for NMR studies, can be purified easily in its natural membrane environment, and has a long shelf-life. Selective labeling of specific amino acids (Kamihira et al. 2005; Mason et al. 2004; Saito et al. 2006) and the retinal (Smith et al. 1989) has led to the assignment and study of selected bR sites, which has provided significant insights into the photocycle (reviewed in Herzfeld et al. 2002; Saito et al. 2007). On the other hand, uniform or extensive isotope labeling of bR has the benefit of obtaining multiple assignments thus observing numerous residues at the same time. However, until recently the study of such a large protein with uniform labeling and membrane embedded has been challenging.

Bacteriorhodopsin was U$-^{13}$C, ^{15}N enriched (Varga et al. 2007a) and purified (Oesterhelt and Stoeckenius 1974) as described previously. The purple membranes were purified on sucrose gradient, washed with MilliQ water, and resuspended in 20 mM sodium citrate buffer at pH 6.0 containing 0.01% NaN_3. The membranes for NMR studies were collected by centrifugation. 2D ^{13}C$-^{13}$C DARR (Takegoshi et al. 2001) homonuclear correlation spectra of bR in purple membranes were collected on a Varian/Magnex Infinity Plus 18.8 T (^{1}H frequency = 800 MHz) solid state NMR spectrometer equipped with a 3.2 mm HCN Balun probe (at 10.776 kHz magic angle spinning frequency and −10°C VT-controlled temperature). Three spectra were collected with various mixing times (3, 15, and 30 ms) which permitted the distinction of one, two, and three bond crosspeaks. 2D ^{13}C$-^{13}$C homonuclear spectra were used to assess sample quality (i.e. homogeneity) and to confirm secondary structure elements. The resolution of these spectra is often indicative of the feasibility of site-specific assignments.

Figure 1 shows the aliphatic–CO and the aliphatic–aliphatic regions of the 15 ms 2D ^{13}C$-^{13}$C DARR spectrum. Spectral quality can be assessed by its signal-to-noise and resolution. In the DARR spectrum of 15 ms mixing time acquired with 64 scans, the signal-to-noise is typically between 10 and 20 for cross-peaks with one bond transfer, although some Ala Cα–Cβ cross-peaks have a signal-to-noise ratio over 20. Cross-peaks of two and three bond transfers have signal-to-noise ratios of 6–12 and 5–7, respectively. The line-widths of isolated peaks are 0.5–0.55 ppm, which indicates a well folded membrane protein and high order of 2D crystals. α-helical membrane proteins tend to have crowded, overlapping spectra due to the similar chemical environment induced by the helices. Indeed, the α-helical nature of several peak groups of overlapping cross-peaks (i.e. Val, Ala, Leu) could be confirmed from chemical shift statistics (Zhang et al. 2003). On the other hand, there are many isolated peaks (including Val, Ala, Leu), which may represent residues with different environments, possibly in the loop regions. The amino acid group with the most striking resolution is isoleucine. Isoleucine sidechains can be easily identified in 2D ^{13}C$-^{13}$C DARR of uniformly ^{13}C, ^{15}N enriched proteins due to their extreme upfield position. Figure 2 shows the connectivity of two Ile residues in the aliphatic region of the DARR spectrum with 15 ms mixing time. The bR amino acid sequence contains 15 Ile residues, out of which at least 12 can be readily resolved in the DARR spectra. Two others could be tentatively assigned, but since the peaks are shifted from the typical Ile region, their identity cannot be confirmed without acquiring additional spectra. Some Ile residues may be also missing due to dynamics or may exhibit more than one resonance due to the co-existence of different conformations, which may be possible in the loop regions where the protein chains are less ordered and more mobile in general. The resolution of the Ile region enabled a residue count, and since approximately the correct number of residues can be counted (12 out of 15), it implies that at least for most parts of the protein, there is a single conformation. This agrees with solution NMR studies of bR in the dark adapted form where a single set of peaks were observed for most of the bR except for the E–F loop (Schubert et al. 2002) and the photoreaction center (Patzelt et al. 2002).

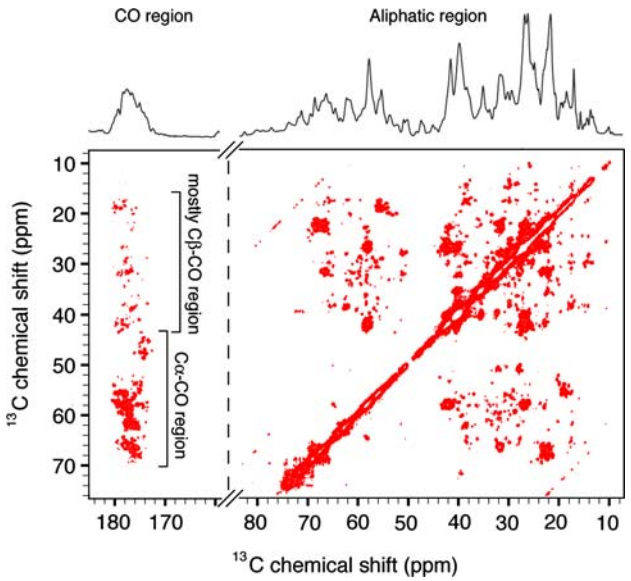

Fig. 1 2D ^{13}C$-^{13}$C DARR spectrum (15 ms mixing time) of uniformly ^{13}C, ^{15}N enriched bR (aliphatic–CO and the aliphatic–aliphatic regions). Besides the one bond transfers, cross-peaks arising from two and three bond transfers can be also identified. For illustration, the one bond Cα–CO and the two bond Cβ–CO cross-peak regions are highlighted. The typical line-widths of isolated resonances were 0.50–0.55 ppm

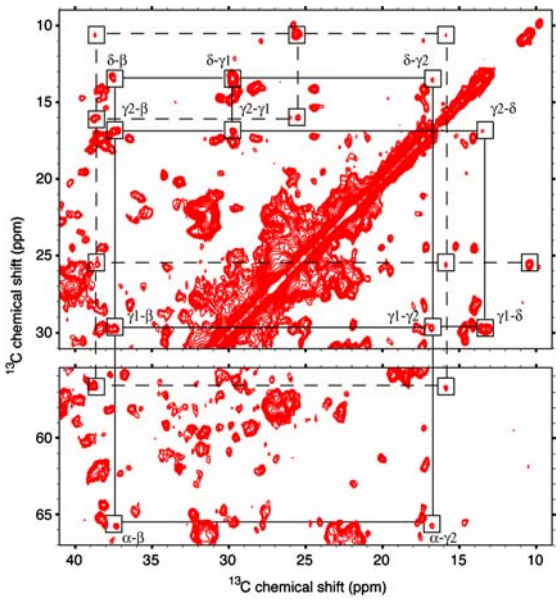

Fig. 2 Connectivity of two isoleucine residues in the 2D $^{13}C-^{13}C$ solid state NMR DARR spectrum (15 ms mixing time) aliphatic region. Crosspeaks of two isoleucine residues are highlighted with boxes and connected with solid and staggered lines respectively for illustration. The crosspeaks for one of the residues are also labeled according to the carbons giving rise to the crosspeak

The signal-to-noise and resolution of the spectra obtained here suggest that site-specific assignments will be feasible with the increased resolution of 2D and 3D heteronuclear spectra and will allow for the more detailed and global study of protein dynamics and the photocycle. As has also been shown that partial, or extensive deuteration leads to noted peak multiplicity for bR (Varga et al. 2007a), with no improvement of line-widths, and therefore fully protonated growth medium was used in this study. Solid state NMR is rapidly developing, and with the availability of high magnetic field (18.8 T), which correlates both with increased sensitivity and resolution, it holds the potential for studying other membrane proteins in the natural or model membrane environment, where essential lipids for activity are present.

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