Solid-State NMR Spectroscopy

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Secondary Structure, Dynamics, and Topology of a Seven-Helix Receptor in Native Membranes, Studied by Solid-State NMR Spectroscopy**

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With the use of solid-state NMR (ssNMR) under magic-angle spinning (MAS) conditions,[1] considerable progress in the investigation of ligand binding to membrane proteins has been achieved.[2–7] However, the determination of entire 3D structures of larger membrane-embedded proteins has been complicated by the length of the amino acid sequence, the high repetitiveness of hydrophobic residues, and the limited spectral resolution arising from the dominant influence of a single type of regular secondary structure (α helix or β sheet).[8–10] In principle, spectral crowding can be reduced by advanced labeling approaches,[9–11] but these may be precluded by low protein expression levels and/or the costs of labeled starting materials.

Herein, we show that uniform isotope labeling (with ^{13}C and ^{15}N), which is straightforward in many cell-based expression systems, the addition of a well-selected set of unlabeled amino acids (reverse labeling),[12,13] and the application of ssNMR spectroscopic methods that separate the signals of mobile, static, and water-exposed protein segments can be used to investigate the structure and topology of an entire seven-helix receptor. The experiments are carried out on a single isotope-labeled sample in a native membrane environment.

Our approach is demonstrated for sensory rhodopsin II from *Natronomonas pharaonis* (NpSRII). NpSRII is a seven-helix (A–G) membrane protein containing retinal, which is bound to a lysine residue through a protonated Schiff base, as cofactor.[14] 3D structures are available for NpSRII in free and transducer-bound forms, and in ground and light-activated states.[15–19] Figure 1a shows a $^{13}C,^{13}C$ spin diffusion (SD)[20] spectrum of an NpSRII sample that is uniformly labeled, with the exception of the four dominant residue types valine, leucine, phenylalanine, and tyrosine, which occur in natural abundance (U[$^{13}C,^{15}N$\(V,L,F,Y)] NpSRII). The spectra were recorded in proteoliposomes at a ^{1}H resonance frequency of 800 MHz. The observed ^{13}C line width of 0.7 ppm is not only indicative of a well-folded membrane protein, but also confirms that high-resolution ssNMR spectra can be obtained for membrane proteins in their natural lipid environment. The reverse labeling of four dominant residue types, which account for 34% of the entire amino acid sequence, significantly improves spectral resolution, compared to that in studies of a uniformly labeled sample.[8] This aspect is particularly striking in Figure 1b, where a 2D SD spectrum recorded under weak coupling conditions (SDWC)[21] reveals a variety of sequential correlations, which are characterized by signal intensities that are significantly higher than the noise level (see Supporting Information). These correlations reduce the level of ambiguity introduced by the occurrence of shorter isotope-labeled amino acid stretches in U[$^{13}C,^{15}N$\(V,L,F,Y)] NpSRII (compared to a fully labeled sample). The correlations were combined with the results of NC (NC = $^{15}N,^{13}C$) dipolar (through-space) transfer experiments[22] to derive sequential resonance assignments, which were classified into three levels of reliability (see Supporting Information). On the basis of these assignments, conformation-dependent chemical shifts could be defined, and these revealed a mainly α-helical conformation for the transmembrane segments of NpSRII.

To further characterize the supramolecular assembly of NpSRII in native membranes, we performed a water-edited $^{13}C,^{13}C$ correlation experiment. The corresponding ssNMR pulse sequence relies on the possibility of polarization exchange between mobile protons from water and protons from the protein complex.[23] Firstly, the signals of the mobile water protons can be selected using a relaxation filter. During a subsequent mixing time, polarization transfer to the immobilized biomolecule can take place. For short mixing times, the resulting polarization-transfer characteristics in the spectra are sensitive to the distance between a given nuclear spin in the interior of the molecular complex and the surrounding water environment.[24]

For the purpose of our studies, we extended the existing pulse schemes by an additional $^{13}C,^{13}C$ mixing unit, which permits a 2D correlation map of all detectable protein resonances to be recorded for a given diffusion time (see

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

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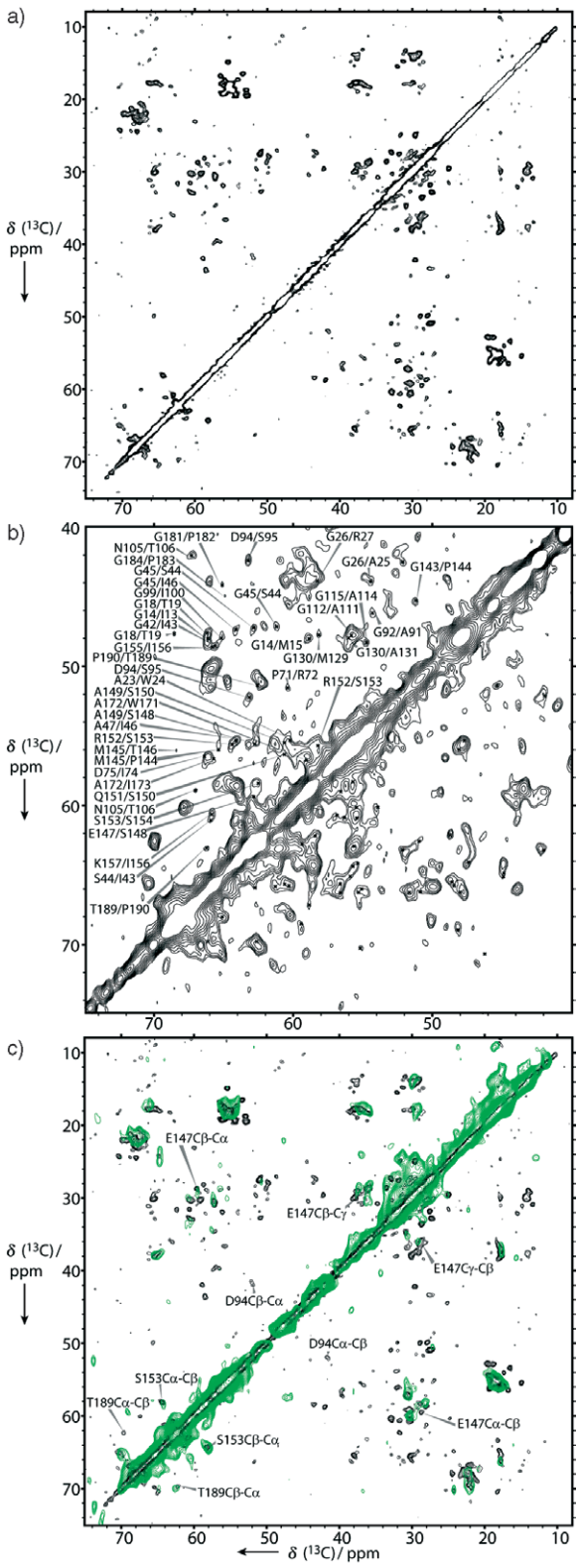

Figure 1. Dipolar $^{13}C,^{13}C$ correlation spectra of U[$^{13}C,^{15}N$\(V,L,F,Y)] NpSRII in proteoliposomes. a) $^{13}C,^{13}C$ SD spectrum (mixing time 15 ms). b) $^{13}C,^{13}C$ SDWC spectrum[21] (mixing time 150 ms); sequential Cα,Cα and Cα,Cβ correlations are indicated. c) Comparison of a water-edited $^{13}C,^{13}C$ correlation spectrum (green; $^{1}H,^{1}H$ mixing time 4 ms) to spectrum (a; black); correlations discussed in the text are indicated.

Supporting Information). The water-edited $^{13}C,^{13}C$ spectrum shown in green in Figure 1c contains only a subset of the correlations observed in Figure 1a. Under the selected experimental conditions, with a $^{1}H,^{1}H$ mixing time of 4 ms, polarization transfer is largely confined to the protein–water interface of NpSRII. Because rigid components are selected by the dipolar polarization transfer, signal sets are observed for immobilized protein segments, such as Glu147, Ser153, and Thr189. Notably, the absence of cross peaks for Asp94 in the water-edited $^{13}C,^{13}C$ correlation experiment suggests that the peptide loop (L3) connecting helices C and D is deeply embedded in the membrane. These correlations allow the known crystal structure of NpSRII to be positioned relative to the lipid–water interface (see below).

The absence of larger fractions of receptor loops in the dipolar spectra shown in Figure 1 may either be due to static structural heterogeneity or fast molecular motion. To discriminate between the two mechanisms, we performed a series of 2D correlation experiments under MAS conditions with scalar (through-bond) transfer units, which detect mobile protein segments.[25] In Figure 2a, the spectra recorded for $^{1}H,^{13}C$ INEPT (red) and $^{1}H,^{13}C$ INEPT-TOBSY (black) transfer experiments are superimposed. Using these spectra, amino acid specific assignments can readily be made. To obtain sequential resonance assignments, we performed NCA and NCOCA correlation experiments based on scalar coupling (Figure 2b,c). Similarly to spectra based on dipolar transfer (Figure 1), the resolution in both the ^{15}N and the ^{13}C dimensions of the NCA and NCOCA spectra is well below

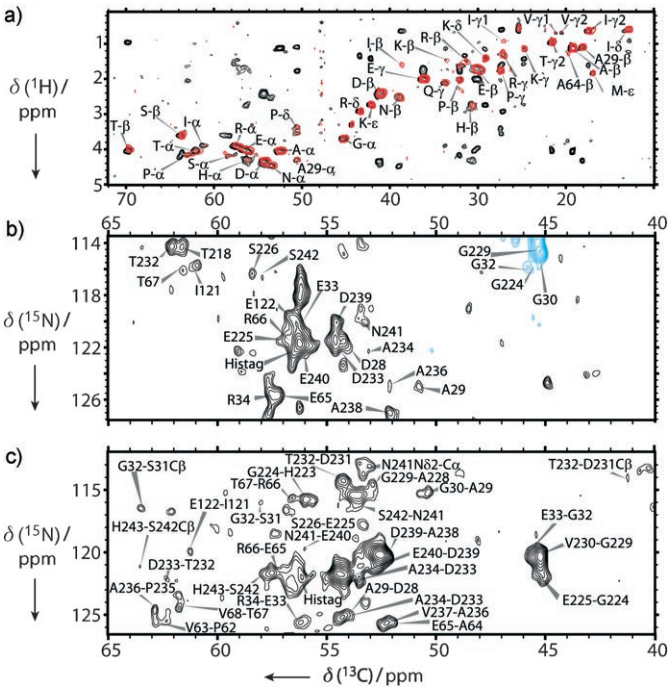

Figure 2. Scalar $^{1}H,^{13}C$ and $^{15}N,^{13}C$ correlation spectra of U[$^{13}C,^{15}N$\-(V,L,F,Y)] NpSRII in proteoliposomes. a) Comparison of a $^{1}H,^{13}C$ INEPT spectrum (red) with a $^{1}H,^{13}C$ INEPT-TOBSY spectrum (black). b) An NCA spectrum;[25] correlations involving glycine residues are indicated in blue. c) An NCOCA spectrum.[25] In all cases, mobile protein segments are detected.

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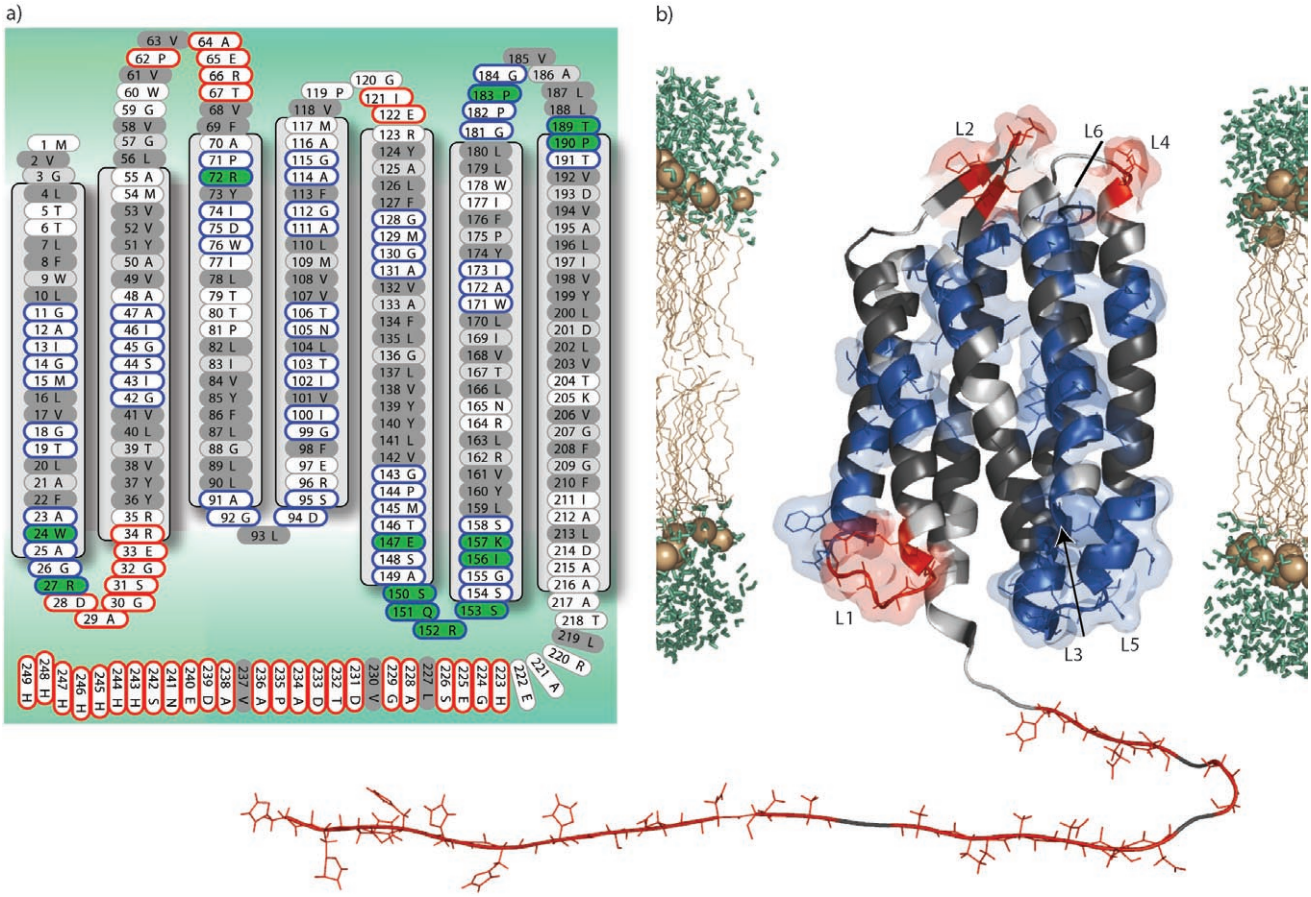

Figure 3. Summary of the results of our ssNMR spectroscopic study on U[^{13}C,^{15}N\(V,L,F,Y)] NpSRII in proteoliposomes, mapped onto a) a schematic of the primary sequence, in an aqueous environment, and b) a representation of the 3D crystal structure of NpSRII,[16] in a lipid-bilayer environment. In both cases, mobile residues are indicated in red, and rigid protein segments in blue. In (a), the residues identified in our water-edited ^{13}C,^{13}C correlation spectrum are indicated in green, residues not labeled in the U[^{13}C,^{15}N\(V,L,F,Y)] NpSRII sample in dark gray, and residues that cannot be assigned sequentially, as a result of the reverse labeling, in light gray.

1 ppm and, thus, permits sequential assignments in a variety of protein segments. Note that in Figure 2b the correlations involving glycine residues (blue) are negative, owing to the specific transfer characteristics within methylene groups. Taking into account the reverse isotope-labeling pattern of our NpSRII sample, the scalar transfer experiments unambiguously reveal that the C terminus, from residue 223 onwards, is mobile. Additional correlations in the 2D spectra suggest that other protein segments also exhibit high mobility. According to our data, these segments involve loops A–B (L1), B–C (L2), and D–E (L4). Indeed, the corresponding secondary chemical shifts of these residues are largely of random-coil character (see Supporting Information), indicating fast structural rearrangements in the protein on a ns-to-μs time scale.

In Figure 3, the results of our ssNMR spectroscopic study are summarized in a schematic of the primary sequence and in a representation of the 3D crystal structure of NpSRII (PDB 1H68).[16] In both cases, residues exhibiting fast motion are indicated in red. Immobilized protein segments that are largely of α-helical character are indicated in blue. In total, resonances were assigned for 98 amino acids, that is, 73% of the sequentially assignable receptor residues. Our data suggest that in NpSRII, reconstituted in purple membrane lipids, only three out of six peptide loops, as well as the C terminus, exhibit sizable dynamics. Furthermore, information from our water-edited ^{13}C,^{13}C correlation experiment was used to position the protein in a model membrane (Figure 3b).

In summary, we have demonstrated that high-resolution ssNMR spectroscopy can be used to study the secondary structure, dynamics, and membrane topology of an entire seven-helix receptor in a native membrane environment. The structural accuracy could be further improved by combining the spectroscopic results for several differently reverse-labeled protein samples, possibly in the context of 3D NMR spectroscopy. The data presented here provide a basis for further ssNMR spectroscopic studies, for example, the use of indirectly detected proton–proton contacts,[7] to assemble the 3D structure of NpSRII in a native membrane environment. Complemented by other biophysical methods,[18,19,26] ssNMR spectroscopic studies, such as those described herein, may provide important insight into the structural details associated with signal transduction in NpSRII or other membrane proteins.

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Experimental Section

U[^{13}C,^{15}N\(V,L,F,Y)] NpSRII was expressed in *E. coli* grown in ^{13}C-labeled glucose and $^{15}NH_4Cl$. Unlabeled amino acids were added in concentrations of 1 mM (leucine, phenylalanine, and tyrosine) or 4 mM (valine). Proteoliposomes were prepared as described in reference [27]. All NMR spectroscopic experiments were conducted using 4-mm triple-resonance (^{1}H,^{13}C,^{15}N) probeheads at static magnetic fields of 18.8 and 14.1 T, corresponding to ^{1}H resonance frequencies of 800 and 600 MHz (Bruker Biospin, Karlsruhe). Dipolar transfer experiments involved broad-band ^{1}H,^{13}C and chemical-shift-selective[22] ^{15}N,^{13}C cross-polarization (CP) schemes. SPINAL64 proton decoupling[28] was applied during the dipolar correlation experiments using radio-frequency fields of 75–90 kHz. Sequential ^{15}N,^{13}C resonance assignments were made by combining 2D NCACX and NCOCX[22] results with the results of ^{13}C,^{13}C correlation experiments performed under weak coupling conditions.[21] MAS rates of 8–12.5 kHz and probe temperatures of −13–5 °C were used. Mobile protein segments were investigated by NMR spectroscopic methods described elsewhere.[25] Water-edited ^{13}C,^{13}C correlation experiments were conducted using a ^{1}H relaxation filter of 1 ms and a ^{1}H,^{1}H mixing time of 4 ms (see Supporting Information).

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