Solid-State NMR Spectroscopy

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# Characterization of Membrane Proteins in Isolated Native Cellular Membranes by Dynamic Nuclear Polarization Solid-State NMR Spectroscopy without Purification and Reconstitution**

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Structural information is key for understanding biological processes. Insoluble proteins, like membrane proteins and amyloid fibrils, are a large class of proteins that are underrepresented in the protein data bank (PDB). As of today, only 7% of all entries in the PDB refer to either a membrane protein or an amyloid fibril structure (membrane protein: 4994 entries; amyloid fibril: 67 entries; total number of entries: 70,303; http://www.rcsb.org/pdb/home/home.do). Given the fact that many drugs target membrane proteins, involved in signal transduction,[1] structural information is highly desirable for a better understanding of the underlying biochemical mechanisms.

The preparation of membrane protein samples with suitable quality for structural biology studies is a tedious task typically involving purification, detergent solubilization, and reconstitution using the correct lipid composition. In particular, the reconstitution step may lead to erroneous results in cases where the protein is not functional when reconstituted into the membrane. We suggest to investigate membrane proteins in their native cellular environment using isolated native cellular membranes containing over-expressed membrane proteins (Figure 1 A). NMR spectroscopy is an intrinsically insensitive method, so higher sensitivity is required to make these native membrane proteins amenable for solid-state NMR investigations. Using dynamic nuclear polarization (DNP) experiments, a substantial nuclear polarization enhancement is achieved by transfer of the magnetization from unpaired electrons to the nuclei.[2,3] In principle, a sensitivity increase of up to 660 is possible with a complete polarization transfer to nuclear spins using this method. In practice, however, the enhancement factor ranges from 20 to 50 times the standard NMR experiment. We show here that the spectral quality (resolution) that is achieved in nonfrozen samples can also be obtained under DNP conditions.

This approach avoids many of the complications encountered today in the field of structural biology of membrane proteins. As a model system, we have chosen the protein Mistic (membrane-integrating sequence for translation of integral membrane protein constructs), which is hypothesized to chaperone cargo proteins to the bacterial lipid bilayer and which folds autonomously into the lipid bilayer.[4] To date, it is still disputed whether Mistic is an integral membrane protein or membrane-associated. The presented approach is valid for both classes of membrane proteins as long as the protein is structurally homogenous when embedded in the lipid bilayer.

E. coli is first grown in unlabeled LB medium. Prior to induction, cells are softly harvested and resuspended in M9 minimal medium containing either $^{15}$N-$NH_4Cl$/u-$^{13}$C glucose, or $^{15}$N-$NH_4Cl$ and u-[$^{13}$C,$^{15}$N]-Gly, Ser, Thr and Val in addition to unlabeled glucose and complementary amino acids.[5] These samples will be hereafter referred to as the u-$^{13}$C-Mistic and GSTV-Mistic samples, respectively. The cells were lysed by a french press. The lipid membranes were separated from the DNA, RNA, and inclusion bodies (20000 g) by multiple steps of low-speed centrifugation. The membranes were collected after an ultra-centrifugation step at 150000 g. Approximately 25 mg of wet membranes were then packed into a 3.2 mm MAS rotor. According to a quantitative analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the sample contains approximately 0.28 mg of Mistic (see Figure S1 in the Supporting Information). In all samples, 20–40 mM of TOTAPOL[6] was added as a stable biradical needed for DNP. No glycerol was added as the high lipid content substitutes for glycerol to create a glass transition state under experimental conditions.[7,8] Under these conditions, magnetization is typically observed to increase by 20–30 times. For comparison, spectra

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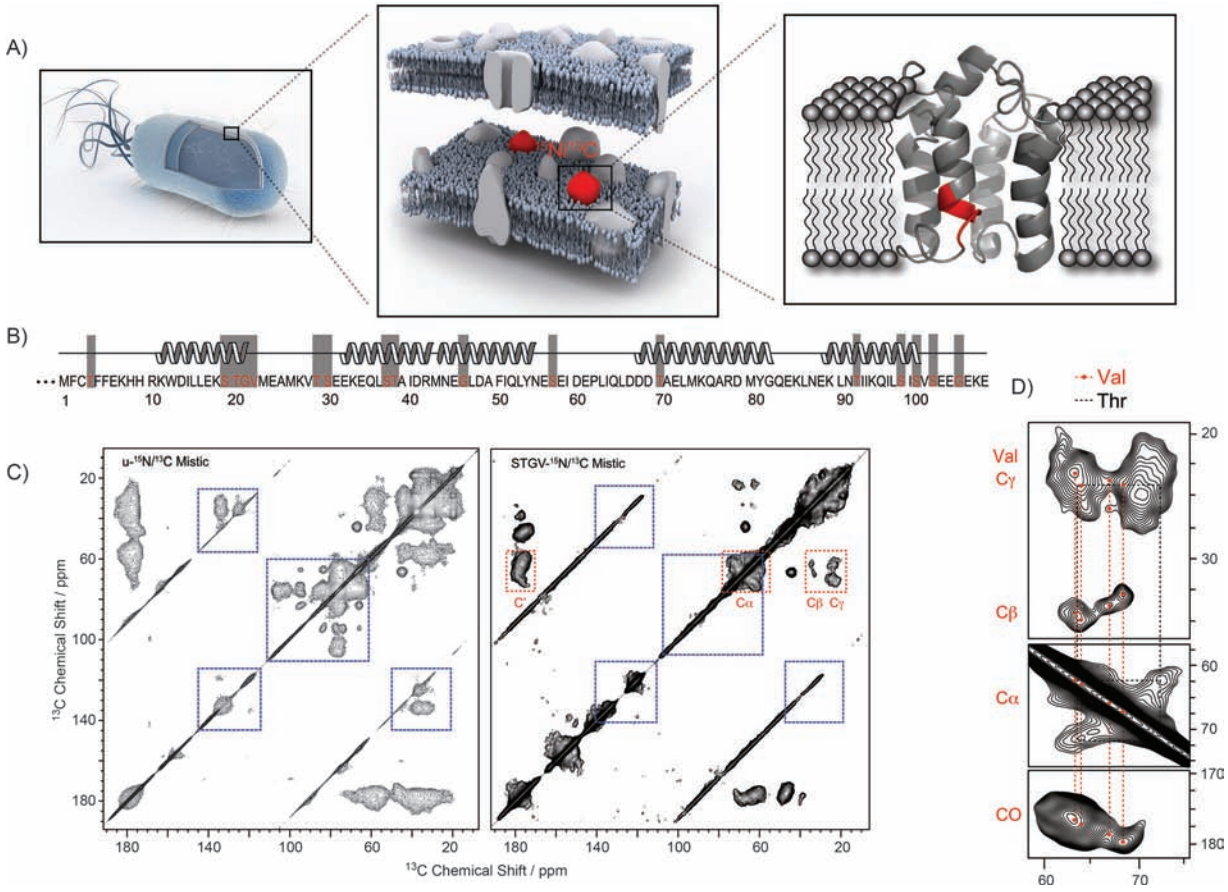


***Figure 1.*** A) For the DNP solid-state NMR experiments, membranes are directly isolated from E. coli cultures in which the membrane protein is over-expressed. As a model system, the protein Mistic is chosen (PDB code: 1YGM). By mass, Mistic accounts for 5% of the total membrane content. The amount of Mistic with respect to the total protein content is on the order of 21%. For solid-state NMR spectroscopy, 25 mg of wet membranes were packed into a MAS solid-state NMR rotor, corresponding to approximately 275 µg Mistic (corresponding to 16 nmol). 3D graphics courtesy of Dr. Barth van Rossum (FMP). In the right panel, consecutive $^{15}$N and $^{13}$C labeling in GSTV-Mistic connecting the residues Ser 20, Thr 21, Gly 22, and Val 23 in α helix 1 is highlighted in red. B) Mistic primary and secondary structure. Isotopically enriched residues in the GSTV-Mistic sample are indicated in red. C) DNP 2D-$^{13}$C,$^{13}$C correlation spectra of membranes extracted from E. coli overproducing Mistic, using u-$^{13}$C-glucose as the sole carbon source (left), and employing unlabeled $^{12}$C-glucose supplemented with the u-[$^{13}$C,$^{15}$N] labeled amino acids Gly, Ser, Thr, and Val (right). D) Enlarged spectral region of the DNP 2D-$^{13}$C,$^{13}$C correlation spectra of GSTV-Mistic, highlighting the valine (red dashed lines) and threonine spin systems. The four expected valine spin systems in Mistic are readily identified in the Cα,Cβ spectral region.

of nonfrozen samples in the absence of TOTAPOL were recorded. The sensitivity of the room-temperature experiments is approximately 30-fold lower, making it impractical to record 3D experiments in a reasonable amount of time.

Spectra of u-$^{13}$C-Mistic and GSTV-Mistic under DNP conditions are shown in Figure 1 C. The strongest signals in the sample grown in M9 minimal medium originate from lipid components. Phospholipids like phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the most abundant lipids in E. coli.[9,10] In addition, sugar resonances are found in the spectra, which originate from the lipopolysaccharide layer of the outer membrane of E. coli. By contrast, the sample that was supplemented specifically with labeled amino acids only shows no sugar and weak lipid resonances. While 2D $^{13}$C,$^{13}$C experiments are not sufficient to determine sequential assignments, the sample quality can be assessed. The four valine spin systems present in Mistic can be easily identified in the 2D experiment (although not yet assigned). 2D Double quantum to single quantum $^{13}$C,$^{13}$C correlation spectra provide a quick assessment of the sample quality by showing the carbon secondary structure chemical shifts in a straightforward manner (see Figure S2 in the Supporting Information).

$^{15}$N-edited 3D experiments like NCACX and NCOCX are an efficient filter to suppress lipid resonances. 3D NCACX and NCOCX experiments of GSTV-Mistic were performed to determine the feasibility for de novo backbone assignments for uniformly isotopically enriched proteins under DNP conditions. Supplementing the growth medium with the amino acids glycine, serine, threonine, and valine yields a quartet of consecutively labeled residues (S20-V23), in addition to several triplets, multiple pairs, and very few isolated labeled residues. Figure 2 shows that a sequential walk of the quartet of backbone resonances can in fact be achieved. The secondary chemical shifts for assigned residues in the GSTV-Mistic sample yield chemical shift index (CSI) scores[11] which are indicative for an α helix. This is in agreement with the structure of detergent-solubilized Mistic[4] in which these residues are located in the first α helix.

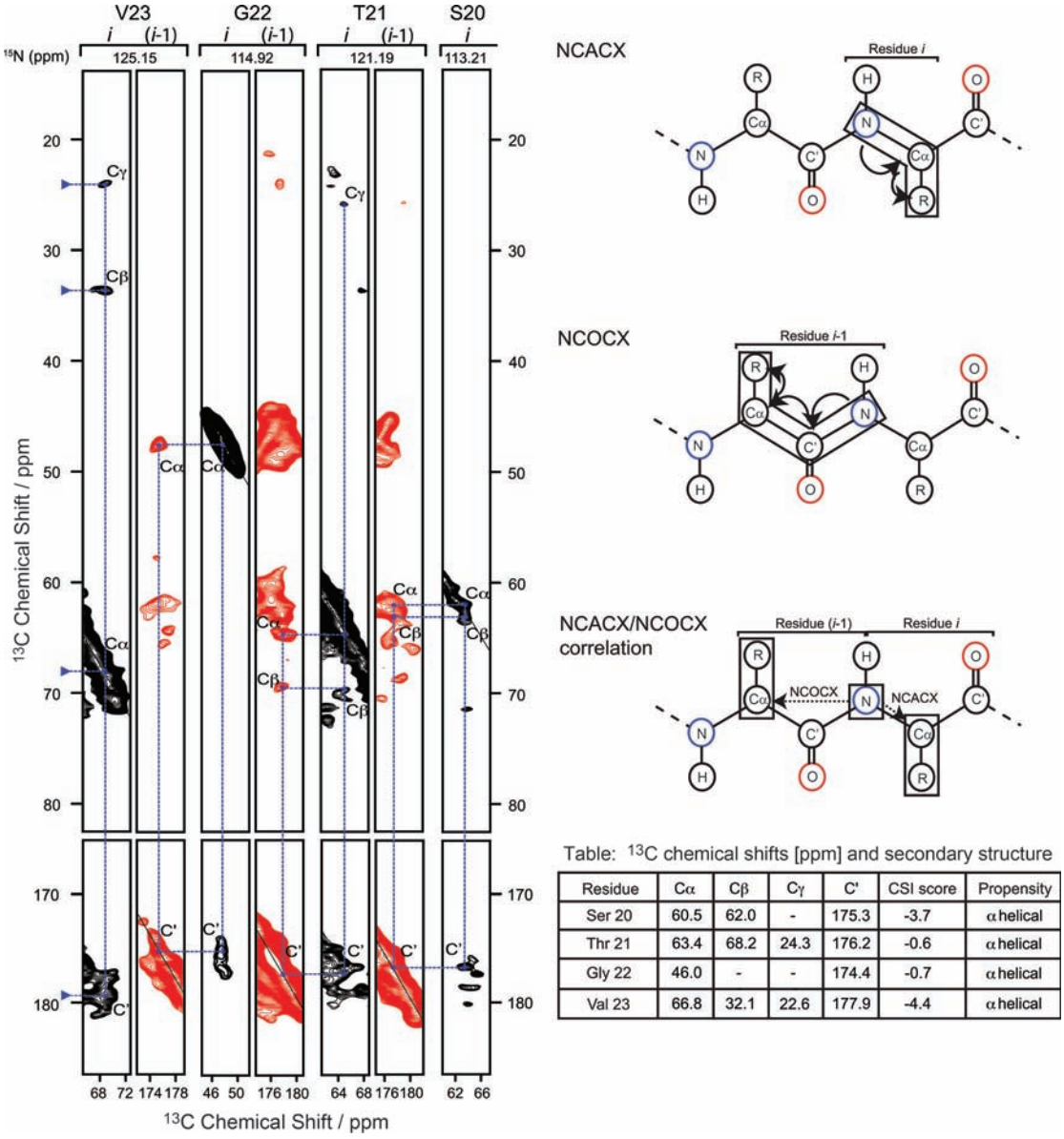


Table: $^{13}$C chemical shifts [ppm] and secondary structure

| Residue | Cα | Cβ | Cγ | C' | CSI score | Propensity |
|---|---|---|---|---|---|---|
| Ser 20 | 60.5 | 62.0 | - | 175.3 | -3.7 | α helical |
| Thr 21 | 63.4 | 68.2 | 24.3 | 176.2 | -0.6 | α helical |
| Gly 22 | 46.0 | - | - | 174.4 | -0.7 | α helical |
| Val 23 | 66.8 | 32.1 | 22.6 | 177.9 | -4.4 | α helical |

***Figure 2.*** 2D strips from the 3D NCACX (black) and 3D NCOCX (red) spectra showing the backbone walk between residues S20 and V23 in Mistic. Correlations expected in the NCACX and NCOCX experiments are schematically depicted on the right (top). Methyl resonances are sometimes difficult to observe at low temperature.[12,13] All spectra were recorded at 100 K and at a magnetic field strength of 9.4 T (400 MHz $^{1}$H Larmor frequency).

We have shown that by using DNP membrane proteins can be investigated in their native cellular environment without the need of purification and reconstitution. The presented approach is cost efficient because a 1/2 L cell culture of isotopically labeled cell media yields roughly four samples. Thus far, all experiments are carried out at 9.4 T (400 MHz, $^{1}$H frequency), which limits the spectral resolution. The DNP enhancement compensates for the line broadening induced by low temperatures by allowing for higher dimensional experiments. With the currently available instrumentation, a gain in resolution can be achieved by performing CX(N)COCX-type experiments[14] which correlate the resonances of the carbon spin system of amino acid (*i*) with the resonances of the spin system of amino acid (*i*−1). This approach avoids evolution of $^{15}$N magnetization, which limits resolution in NCOCX/NCACX-based sequential assignment experiments. Alternatively, deuteration techniques may enable a further increase in sensitivity.[15] Inclusion of a proton chemical shift dimension may improve dispersion of the resonances in the course of the assignment process.[16] In the future, the structural characterization of membrane proteins can be achieved by employing multidimensional CHHC-type experiments in fully or randomly protonated samples.[17,18] In cases where deuteration techniques can be successfully implemented, the availability of long-range distance information might allow further refinement of the membrane protein structure.[19,20]

We believe that the reported approach will have a significant impact on the ability to determine membrane protein structures and to characterize protein–ligand and protein–lipid interactions. In this way, more complex systems that cannot currently be produced and reconstituted in sufficient amounts may become accessible.

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