Solid-State NMR Spectroscopy

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# A Sedimented Sample of a 59 kDa Dodecameric Helicase Yields High-Resolution Solid-State NMR Spectra**

*Carole Gardiennet, Anne K. Schütz, Andreas Hunkeler, Britta Kunert, Laurent Terradot, Anja Böckmann,* and Beat H. Meier**

Solid-state NMR spectroscopy has developed into a useful method for protein structure determination. In contrast to diffraction methods, NMR spectroscopy does not rely on long-range order and, besides microcrystals,[1] viruses,[2] fibrils,[3] membrane proteins[4] and large protein complexes[5] have been investigated. Still, sample preparation is crucial to obtain high-resolution spectra, and resolution and sensitivity remain central issues. We report herein that when a large ($12 \times 59$ kDa) biomolecule is centrifuged as a sediment directly into NMR rotors, highly resolved spectra can be recorded with a good signal-to-noise ratio, rivaling the spectra of carefully crystallized preparations. Bertini and co-workers[6] have recently shown that the NMR rotor acts as an ultracentrifuge and that solutions of large proteins behave like solids when spun at sufficient frequencies; however, sensitivity in this kind of experiment is limited by the protein's solubility[6a] and remains low. We demonstrate herein that the signal-to-noise issue can indeed be resolved by sedimenting a protein solution directly into an NMR rotor using an ultracentrifuge with a specially adapted filling tool, in order to obtain protein densities comparable to those of microcrystalline preparations. Centrifugation directly into NMR rotors has already been employed to increase the concentration of crystalline and fibrillar samples in the past.[7] Here we sediment soluble proteins using a similar method, opening a new avenue for the investigation of samples that are difficult to crystallize or precipitate by addition of crowding agents like polyethylene glycol (PEG). The approach provides sufficient sensitivity for the study of large proteins, as shown here for the 59 kDa DnaB helicase.

DnaB helicases are bacterial ATP-driven enzymes that unwind double-stranded DNA during DNA replication. DnaBs are generally hexameric and form ring-shaped assemblies that encircle single-stranded DNA. In the following we studied DnaB from *Helicobacter pylori*, with a monomer atomic mass of 59 kDa (57 kDa and tag). The crystal structure of the N-terminal domain has been solved (Ref. [8], PDB code 3GXV), and more recently also that of the C-terminal domain of the protein.[9] Biochemical and electron microscopy studies of the full-length protein revealed that it forms, in contrast to the other DnaBs, dodecamers assembled as double hexameric rings.[9] No crystal structure of the full-length protein is available. Crystals of DnaB obtained are so far not suitable for X-ray crystallography studies but, however, for high-quality solid-state NMR spectra (see below). The DnaB dodecamer has an atomic mass of 708 kDa, and alternative sample preparations of the protein are of interest to study its conformation in interaction with DNA or other agents, in the presence of which crystallization has been unsuccessful.

Figure 1 compares the NMR spectra from a crystalline preparation of DnaB obtained from an optimized crystallization buffer with those from a sample obtained by simply sedimenting a protein solution of 49 mg mL$^{-1}$ into a 3.2 mm Bruker rotor at 200000 g. The two spectra are remarkably similar as judged from the overall appearance. An overlay of the full spectra is provided in the Supporting Information. This finding is confirmed by a detailed investigation of the isolated resonances. As an example, the alanine Cα/Cβ region is enlarged in Figure 2a. In addition to many overlapping signals, which correspond to the α-helical region (around $\delta =$ 18/55 ppm), further alanine resonances are seen. DnaB contains six β-sheet alanine residues (in the C-terminal part),[9] for which the SHIFTX[10] predictions are indicated in Figure 2a. These well-resolved resonances are virtually identical for the two preparations. This finding is further confirmed by looking at 1D sections, for example, a trace at $\delta_1 = 71.9$ ppm (Figure 2b).

The signal-to-noise ratios of the spectra of crystalline and sedimented samples are roughly identical (see Figures 1 and 2b); the spectra of the sedimented sample were recorded with 5.5 times more scans, indicating that the rotor of the sedimented sample contains roughly half the material of the rotor with microcrystals and confirming the estimates made from the amount of materials used. The estimated final concentration in the rotor with the sedimented protein is about 200 mg mL$^{-1}$, whereas the concentrated solution of DnaB contains 49 mg mL$^{-1}$. While the protein contents can

[*] Dr. C. Gardiennet, Dr. B. Kunert, Dr. L. Terradot, Dr. A. Böckmann
Institut de Biologie et Chimie des Protéines
UMR 5086 CNRS/Université de Lyon1
7 passage du Vercors, 69367 Lyon (France)
E-mail: a.bockmann@ibcp.fr
A. K. Schütz, A. Hunkeler, Prof. Dr. B. H. Meier
Physical Chemistry, ETH Zürich
Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland)
E-mail: beme@ethz.ch

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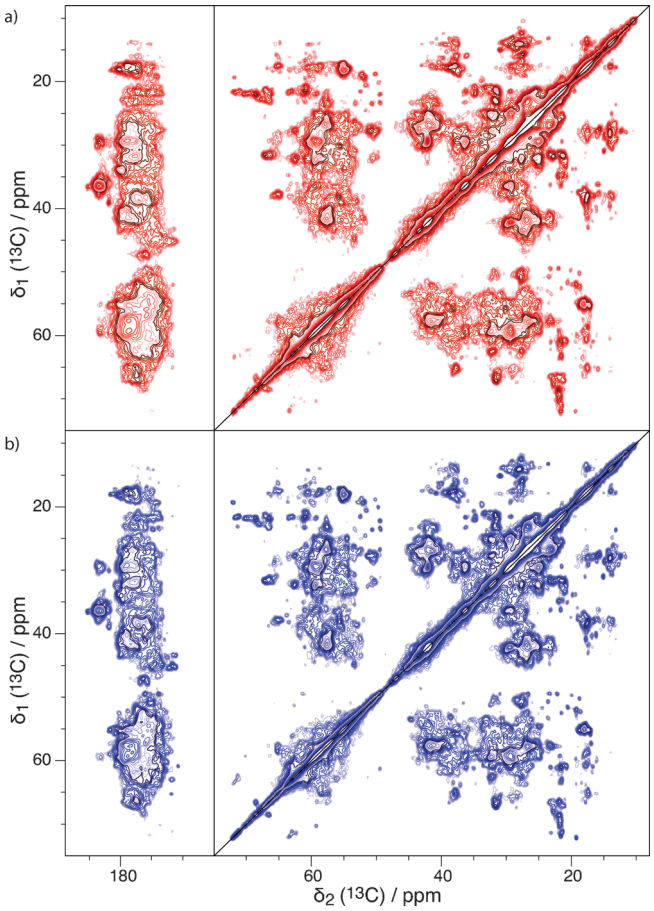


***Figure 1.*** Two-dimensional DARR spectra (50 ms mixing time, 18 kHz MAS) of the 521 residue protein DnaB (molecular mass 59 kDa): a) sedimented (in red) and b) microcrystalline (in blue) DnaB samples. Both spectra feature roughly the same signal-to-noise ratio but spectrum (a) was acquired with 5.5 times more scans, namely 22. In the indirect dimension 2000 increments were acquired in a total measurement time of 7 and 37 h, respectively.

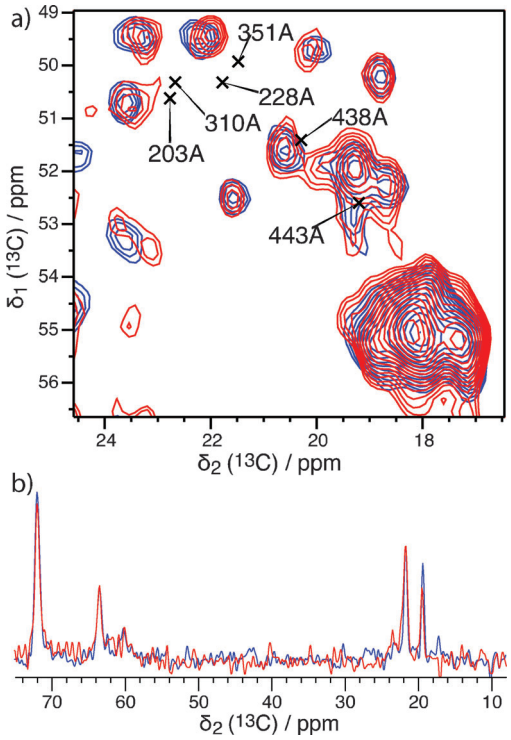


***Figure 2.*** Alanine region of the 2D DARR spectra from Figure 1 (sedimented sample, red; microcrystalline sample, blue). Crosses indicate SHIFTX predictions. b) One-dimensional traces through the same spectra at $\delta_1 = 71.9$ ppm.

probably be further improved by optimizing sample preparation, we note that the first attempts have already led to an amount of protein which is almost half of that of a microcrystalline sample, and to an increase by a factor of 4 when compared to the maximum amount of sample that could be sedimented from a protein solution spinning in the rotor.[6b] Importantly, an increase by a factor of 4 in sample quantity leads to a decrease in measurement time by a factor of 16. This is often the deciding factor in whether it is possible to run 3D spectroscopy needed for sequence assignments, and based on this, carrying out a site-resolved conformational analysis of the protein. We note that after ultracentrifugation the protein sediments in the rotor looks like a gel and can be recovered from the rotor and diluted again. SDS-PAGE analysis shows no proteolysis.

To sediment proteins from solution directly into the NMR rotor we developed a special filling tool, which withstands centrifugal forces up to 210000 g and contains a sufficiently large reservoir (1 mL) to hold the protein solution and enable a considerable concentration in a single step. The volume ratio of roughly 30 between the reservoir and the rotor means that roughly 30 times higher concentrations can be obtained in the rotor than by simply pipetting solution into the rotor.

The centrifugation tools were fabricated from carbon-fiber-reinforced plastic (Sutter Kunststoffe AG, Switzerland) to withstand the centrifugal forces and features a funnel screwed directly onto the top of the MAS rotor (Figure 3). The bottom part is fabricated from a pultruded composite with the fiber direction along the axis. The funnel is made from a wound carbon tube with the fiber direction perpendicular to the axis. The weight of the empty tool was 4 g complying with the specifications for the ultracentrifuge. The tool shown in Figure 3 fits a 3.2 mm Bruker rotor. The tool, as described, can sediment proteins with a (multimer) mass of 100 kDa at 200000 g (and potentially of 25 kDa at 500000 g). Under these conditions, a full rotor can be obtained provided that the initial concentration is in the range mentioned above.

To investigate whether the sediment consists of micro- or nanocrystals which might form while the concentration gradually increases during the centrifugation, we performed X-ray diffraction experiments on the sediment (see the Supporting Information) and detected no crystalline order.

Using a—for NMR spectroscopy—very large protein, namely a dodecamer of 708 kDa ($12 \times 59$ kDa), whose structure has not been solved by X-ray diffraction, we have demonstrated that a high-quality sample for solid-state NMR spectroscopy showing both high resolution and good signal-to-noise ratio can be obtained by ultracentrifugation of the protein solution into an NMR rotor. While high-resolution

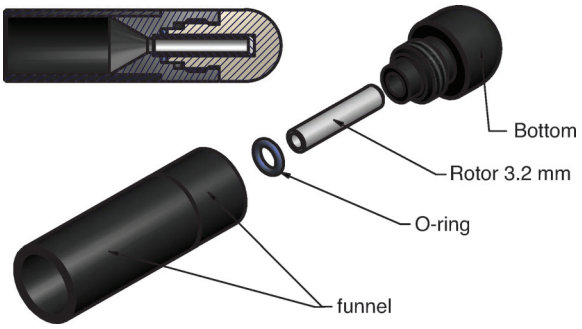


***Figure 3.*** Rotor-filling tool for protein sedimentation using an ultracentrifuge. The NMR rotor is placed into a bottom insert made from carbon-fiber-reinforced plastic (CFRP) and a funnel is screwed on top. The tool shown here fits into the buckets of a swinging rotor (Beckman SW 60 Ti) and was used in a Beckman Optima LE-80K preparative ultracentrifuge. The tool shown fits a 3.2 mm Bruker rotor.

spectra of a sedimented smaller 20 kDa protein have been observed before,[6] we now demonstrate that the necessary sensitivity for large proteins can also be obtained. The spectrum of the gel-like sample of DnaB is virtually identical to that of the microcrystalline form, with some minor shifts of peak positions that could possibly be explained by different "crystal" contacts. The linewidths of both samples are the same and are typical for well-ordered proteins.

The sedimented DnaB sample has shown to be stable at least over several weeks and a spectrum of the sediment stored for a month at 4 °C was identical to the one in Figure 1. For DnaB, this opens the possibility to study this protein in noncrystalline forms, notably bound to DNA, as when loaded, in its natural context, on the replication fork. Also, in a more general way, this kind of sample preparation could be possible for other proteins, as well as large protein complexes, such as membrane proteins or complexes thereof in lipids/detergents, virus capsids, protein/DNA complexes, and prefibrillar aggregates, opening up an entirely new avenue for protein NMR spectroscopy.

## *Experimental Section*

Sample preparation: Uniformly [$^{13}$C,$^{15}$N]-labeled *Helicobacter pylori* DnaB with an N-terminal His tag was overexpressed in *E. coli* using minimal M9-type media supplemented with uniformly $^{13}$C-enriched glucose (2 g L$^{-1}$) and $^{15}$N-labeled ammonium chloride (2 g L$^{-1}$) as the sole carbon and nitrogen sources. The protein was purified using affinity chromatography (5 mL HisTrap HP from GE Healthcare), followed by anion-exchange chromatography (5 mL HiTrap Q HP from GE Healthcare).

For subsequent crystallization, DnaB was concentrated to 16 mg mL$^{-1}$ (Millipore concentrator, 30 kDa cut-off). Crystallization was performed by mixing equal volumes of precipitant (25 % PEG 2k MME, 0.2 M magnesium acetate) and protein in a nine-well plate with 25 mL precipitant solution in the reservoir. Precipitate as well as hexagonal crystals were obtained after one week at 20 °C. Microcrystals were centrifuged into the rotor (65 000 g, 16 h, 4 °C).

For the sedimented protein sample, no anion-exchange step was performed, and the protein was concentrated to 49 mg mL$^{-1}$ in 250 mM NaCl, 40 mM Tris-HCl pH 8.0, 5 % glycerol. The protein solution was directly ultracentrifuged into a 3.2 mm Bruker rotor (200 000 g overnight 4 °C, Beckman rotor SW60.Ti).

The concentrated protein solution for crystallization and for sedimentation was investigated by SEC-MALLS measurements and the solution was found to consist of dodecamers (predominantly) as well as aggregates thereof. Detailed results at lower concentrations are given in Ref. [9]. Electron micrographs are also given in Ref. [9]. The SEC-MALLS data given in the Supporting Information demonstrate that the mass distribution after ultracentrifugation was identical (and similar to that lower concentration) and that no signs of deterioration were present.

Solid-state NMR experiments: NMR experiments were carried out on a Bruker Biospin AVANCE II+ spectrometer operating at 850 MHz $^{1}$H frequency using a 3.2 mm triple-resonance ($^{1}$H,$^{13}$C,$^{15}$N) LLC probe (Bruker Biospin). The sample temperature was estimated using the chemical shift of supernatant water[7e] and set to 281 K. All experiments were conducted at 18 kHz spinning frequency using 90 kHz SPINAL64[11] proton decoupling. For the DARR experiments[12] the proton irradiation was set to 18 kHz. The proton and carbon field amplitudes during the $^{1}$H–$^{13}$C cross-polarization transfer step were 70 and 55 kHz, approximately. The spectra for the crystalline sample were acquired with 4 and 22 scans, respectively, using 2000 increments in $t_1$ which, with an interscan delay of 3 s, led to total acquisition times of 6.7 and 36.7 h, respectively. Spectra were processed with NMRPipe[13] and analyzed and plotted with CCPNmr Analysis.[14]

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