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

Carole Gardienet¹, Anne Schütz², Andreas Hunkeler², Britta Kunert¹, Laurent Terradot¹, Anja Böckmann^{1*} and Beat H. Meier^{2*}

Solid-state NMR has lately developed into a useful method for protein structure determination. In contrast to diffraction methods, NMR 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 demonstrate in this contribution that the sediment of a simple centrifugation of a large (12x59 kDa) biomolecule directly into NMR rotors can lead to highly resolved spectra with good signal-to-noise ratio, rivaling the ones of carefully crystallized preparations. If it has been shown recently by Bertini and coworkers^[6] that the NMR rotor acts as an ultracentrifuge and that solutions of large proteins behave like solids when spun at sufficient frequencies, sensitivity in this kind of experiment is limited by the protein's solubility^[6a], and remains low. In the following, we demonstrate that the signal-to-noise issue can indeed be solved by sedimenting a protein solution directly into an NMR rotor using a ultracentrifuge using a specially adapted filling tool, in order to obtain protein densities comparable to microcrystalline preparations. Centrifugation directly into NMR rotors has already been employed to increase 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 which are difficult to crystallize or precipitate by addition of crowding agents

like PEG. The approach provides sufficient sensitivity for the study of large proteins, as shown here for the 59 kDa DnaB.

DnaB helicases are bacterial ATP-driven enzymes that unwind dsDNA during DNA replication. DnaBs are generally hexameric and form ring-shaped assemblies that encircle ssDNA. 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 the one 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 the presence of DNA or other interactants, in presence of which crystallization has been unsuccessful.

Figure 1 compares the NMR spectra from a crystalline preparation of DnaB from an optimized crystallization buffer with a sample obtained from simply sedimenting a protein solution of 49 mg/mL into a 3.2 mm Bruker rotor at 200'000 g. The two spectra are remarkably similar as judged from the overall appearance, even more so than spectra from "in-MAS-rotor" sedimentation^[6]. An overlay of the full spectra is provided in the Supplementary Information. This finding is confirmed by a detailed investigation of the outlier resonances. As an example the alanine C α /C β region is enlarged in Figure 2a). In addition to many overlapping signals which correspond to α -helical resonances (around 18/55 ppm), further alanine resonances are seen. DnaB contains 6 β -sheet alanines (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 extracts, e.g. a trace at δ_1 =71.9 ppm (Figure 2b).

The signal-to-noise ratio of the spectra of crystalline and sedimented samples is roughly identical (see Figures 1 and 2b), with the one of the sedimented sample having been 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, 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, compared to a concentrated solution of DnaB at 49 mg/mL. While the protein contents can probably be further improved by optimizing sample preparation, we note that the first attempts have already led to a concentration which is almost half 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 often makes the

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difference between being able or not to run 3D spectroscopy needed for sequential 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 stands centrifugal forces up

to 210'000 g and contains a sufficiently large reservoir (1 ml) to hold the protein solution and enabling a considerable concentration in a single step. The volume ratio of roughly 30 between reservoir and rotor allows for roughly 30 times higher concentrations in the rotor than by pipetting solution into the rotor.

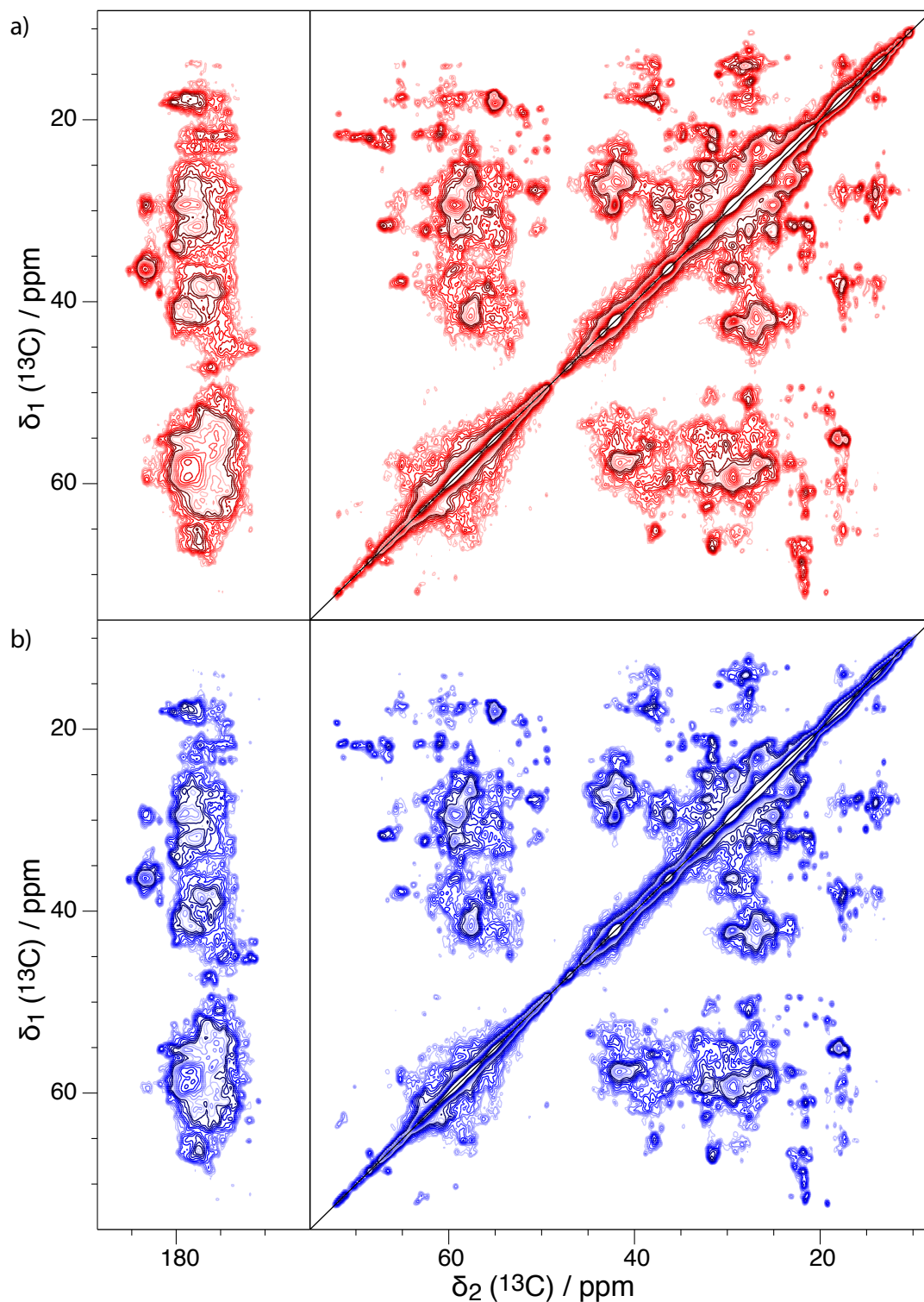


Figure 1. Two-dimensional DARR spectra (50 ms mixing, 18 kHz MAS) of the 521 residue protein DnaB (molecular mass 59 kDa) (a) sedimented (in red) and (b) microcrystalline (in blue) DnaB sample. Both spectra feature roughly the same signal-to-noise ratio but a) has been acquired with 5.5 times more scans, namely 22,000 increments were acquired in the indirect dimension in a total measurement time of 37 h.

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.

To investigate if the sediment consist of micro- or nanocrystals which might form during the gradually increasing concentration during the centrifugation, we have performed X-ray diffraction experiments on the sediment (see Supplementary information) and have detected no crystalline order.

Using a – for NMR spectroscopy – very large protein, namely a dodecamer of 708 kDa (12x59 kDa), whose structure has not been solved by X-ray diffraction, we have demonstrated that a high quality sample for solid-state NMR 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 spectra of a sedimented smaller 20 kDa protein has been observed before^[6], we now demonstrate that also the necessary sensitivity for large proteins can be obtained. The gel-like sample of DnaB has a virtually identical spectrum when compared to the microcrystalline form, with some minor shifts of peak positions that could possibly be explained by different “crystal” contacts. The linewidth of both samples are the same and are typical for well ordered proteins.

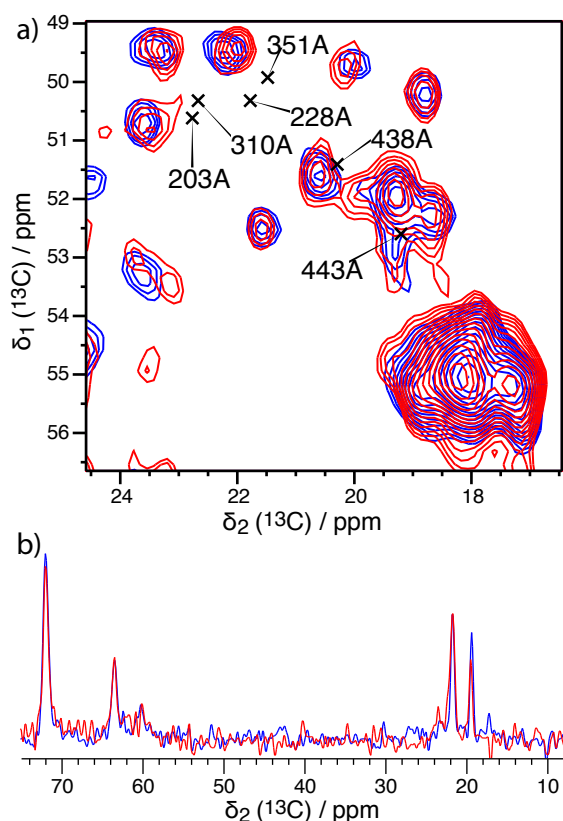


Figure 2. Extract of the alanine Ca/C β crosspeak regions from Figure 1. a) extract of the Alanine region of the 2D DARR spectra (sedimented sample, red; microcrystalline sample, blue). b) One-dimensional traces through the same spectra at $\delta_1=71.9$ ppm.

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 non-crystalline 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, as for example 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.

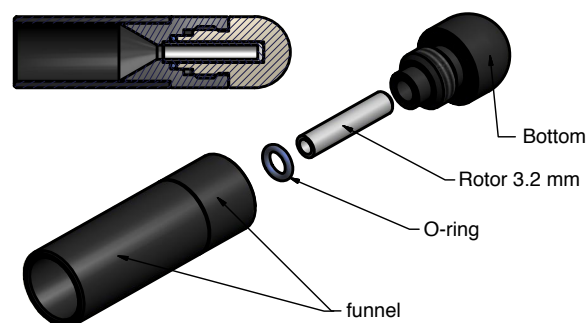


Figure 3. Rotor filling tool for protein sedimentation using an ultracentrifuge. The NMR rotor is placed into a bottom insert made from carbon-fiber-reinforced plastic (CFRP) and a funnel is screwed on top. The tool shown here fits into the buckets of a swinging rotor (Beckman SW 60 Ti) and was used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

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 (2g/L) and ^{15}N -labeled ammonium chloride (2g/L) as 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 (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 9-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 (65000 g, 16h, 4°C).

For the sedimented protein sample, no anion exchange step was performed, and the protein was concentrated to 49 mg/mL 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 (200000g overnight 4°C, Beckman rotor SW60.Ti).

The concentrated solution for crystallization and for sedimentation was investigated by SEC-MALLS measurements and the solution was found to consist of dodecamers (predominately) as well as aggregates thereof. Detailed results at lower concentrations are given in ^[9]. Electronmicrographs are also given in ^[9]. The SEC-MALLS data given in the Supporting material demonstrate that the mass distribution after ultracentrifugation was identical 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 ^1H frequency using a 3.2 mm triple-resonance (^1H , ^{13}C , ^{15}N) LLC probe (Bruker Biospin). 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 ^1H - ^{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 3s leads 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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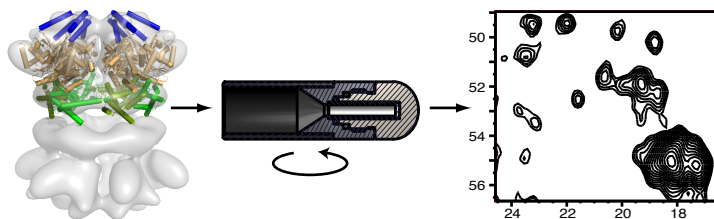
NMR: sedimented protein as good as crystals

Carole Gardiennet¹, Anne Schütz²,
Andreas Hunkeler², Britta Kunert¹,
Laurent Terradot¹, Anja Böckmann^{1*}
and Beat H. Meier^{2*}

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

Getting high-quality NMR samples more easily: Preparing solid-state NMR samples that yield high sensitivity, high-resolution spectra is a time-intensive and complex issue. We demonstrate that a sample of the 59 kDa Protein DnaB, prepared simply by preparative centrifugation leads to spectra that are as good as the ones from carefully grown microcrystals.



Supporting information for:

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

Carole Gardiennet¹, Anne Schütz², Andreas Hunkeler², Britta Kunert¹, Laurent Terradot¹, Anja Böckmann^{1} and Beat H. Meier^{2*}*

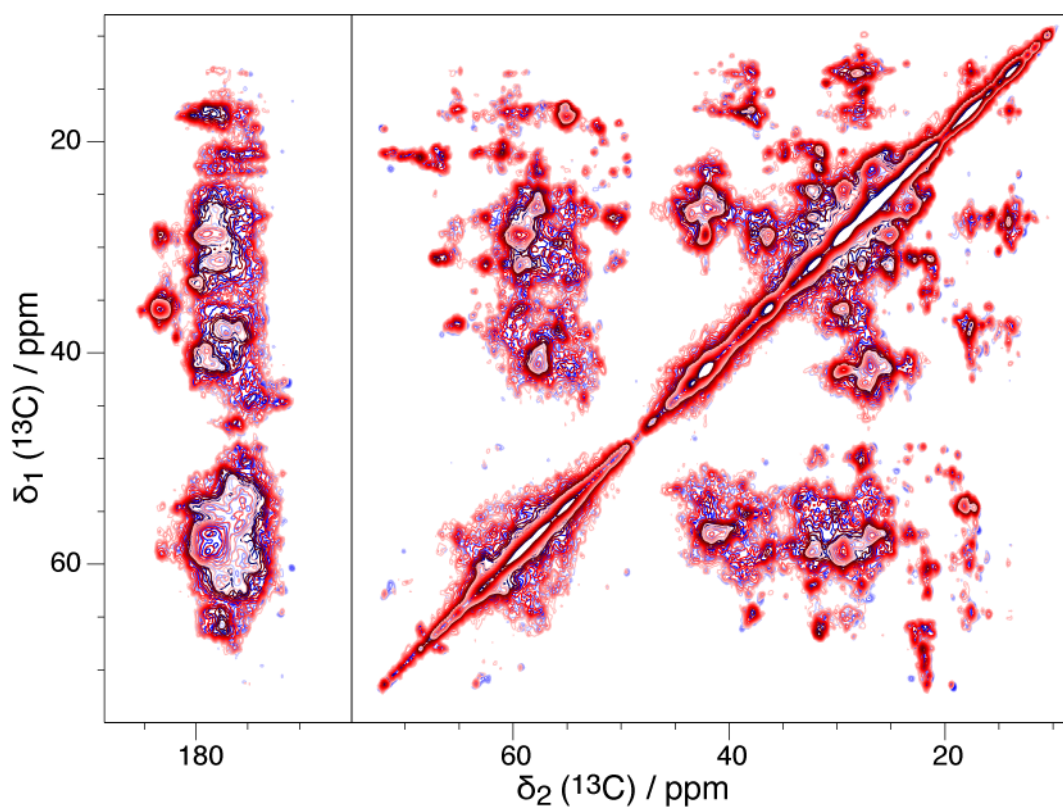


Figure S1. Overlay of the two-dimensional DARR spectra (50 ms mixing, 18 kHz MAS) of the 521 residue protein DnaB (molecular mass 59 kDa) microcrystalline (blue) and sedimented (red) DnaB sample.

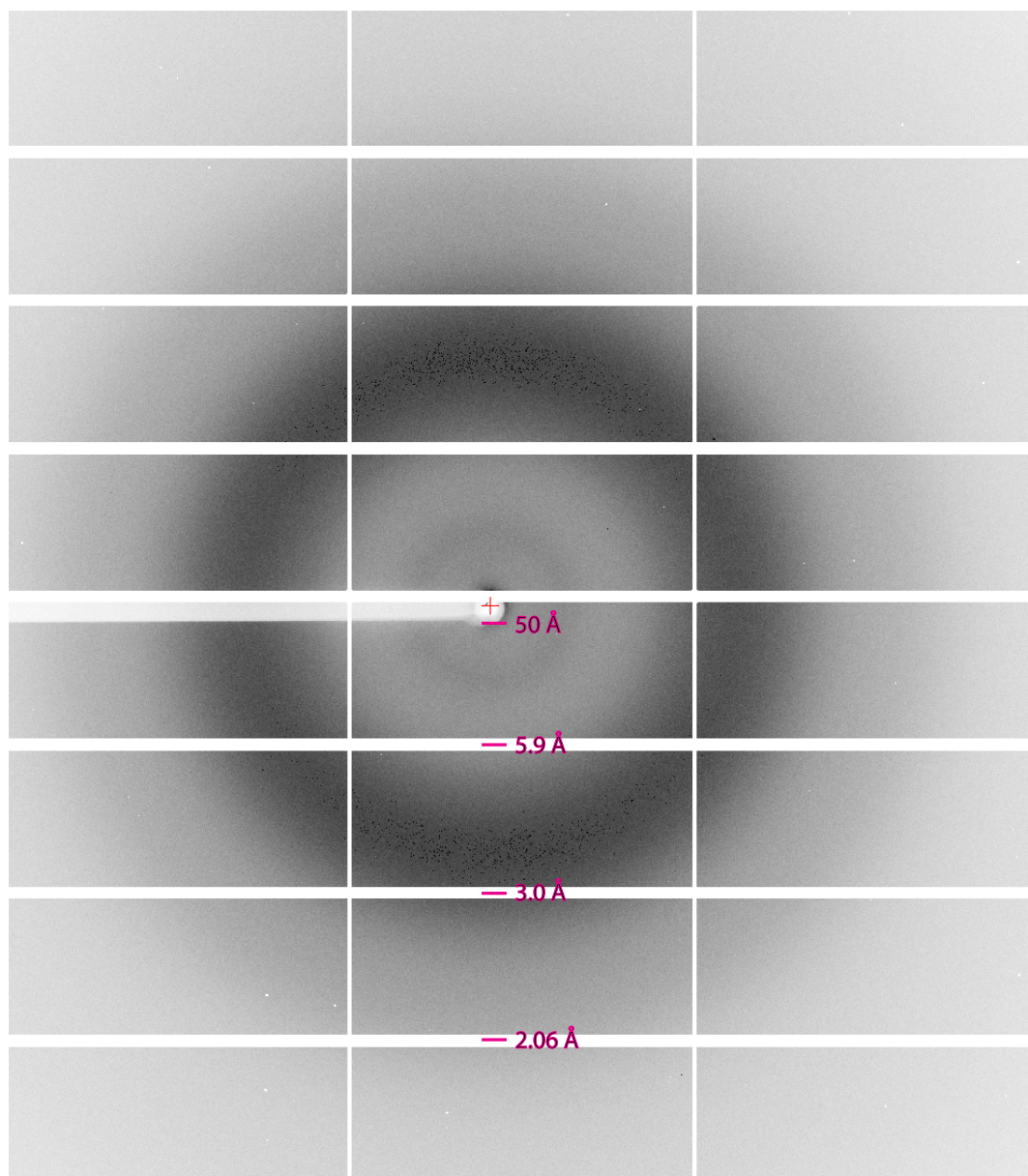


Figure S2. X-ray Diffraction pattern of sedimented DnaB recorded at room temperature the beamline PXI - X06SA of the Swiss Light Source (Pilatus detector) at a wavelength of 1.00004 Å. No micro- or nano-crystals were detected and no long-range order is present. For typical patterns of micro- or nanocrystalline samples as used in MAS NMR experiments see (Martin, R., & Zilm, K. (2003). *Journal Of Magnetic Resonance*, 165(1), 162–174.)

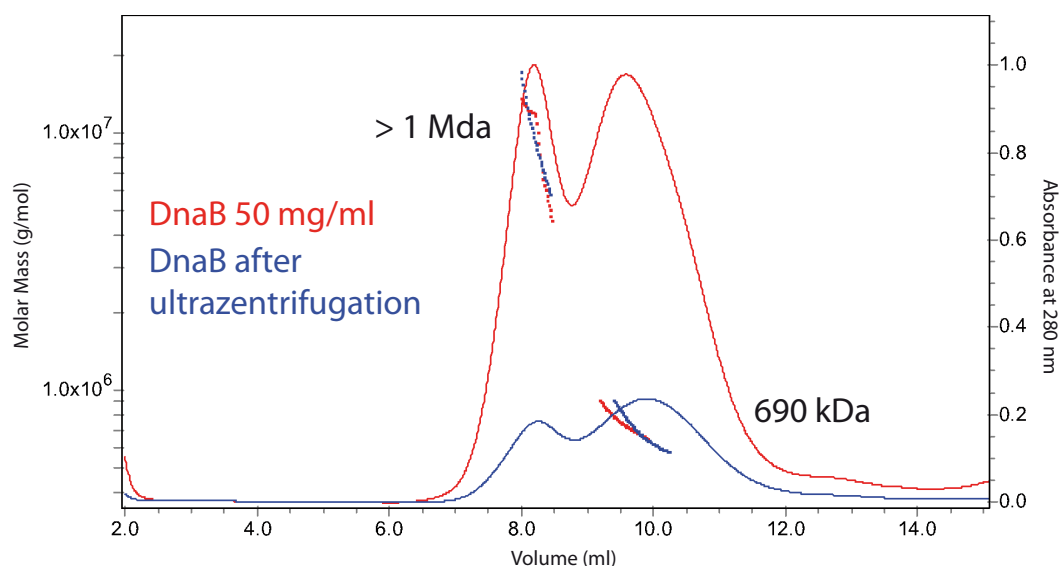


Figure S3. Chromatograms obtained from SEC-MALLS measurements with DnaB at a concentration of 50 mg/ml (red), and with resuspended sediment after ultracentrifugation (blue) at a concentration of 5 mg/mL in SEC buffer (40 mM Tris [pH 8], 150 mM sodium chloride, 5% glycerol and 1mM dithiothreitol). Molar mass (Da) points for the two peaks are indicated. The first peak corresponds to HpDnaB aggregation (heterogeneous peak containing assemblies of dodecamers), whereas the second peak has a mass of around 680 kDa, corresponding to HpDnaB dodecamers. Light absorbance at 280 nm is indicated by the continuous lines. The data are very similar to the ones obtained at lower concentration (described in detail in {Stelter, 2012 #9946}) and indicate the integrity of the protein at high concentration and after sedimentation (see also the electronmicrographs in this reference).