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SedNMR: On the Edge between Solution and Solid-State NMR

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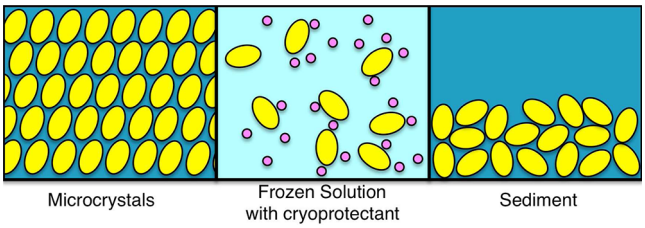

Solid-state NMR (SS-NMR) of proteins requires that those molecules be immobilized, usually by crystallization, freezing, or lyophilization. However, self-crowding can also slow molecular rotation sufficiently to prevent the nuclear interactions from averaging. To achieve self-crowding, researchers can use a centrifugal field to create a concentration gradient or use regular ultracentrifugation to produce highly concentrated, gel-like solutions. Thus sedimented solute NMR (SedNMR) provides a simple method to prepare biological samples for SS-NMR experiments with minimal perturbation. This method may also give researchers a way to investigate species that are not otherwise accessible by NMR. We induce the sedimentation in one of two ways: (1) by the extreme centrifugal force exerted during magic angle spinning (MAS-induced sedimentation or *in situ*) or (2) by an ultracentrifuge (UC-induced sedimentation or *ex situ*).

Sedimentation is particularly useful in situations where it is difficult to obtain protein crystals. Furthermore, because the proteins remain in a largely hydrated state, the sedimented samples may provide SS-NMR spectra that have better resolution than the spectra from frozen solutions or lyophilized powders. If sedimentation is induced *in situ*, the same protein sample can be used for both solution and SS-NMR studies.

Finally, we show that *in situ* SedNMR can be used to detect the NMR signals of large molecular adducts that have binding constants that are too weak to allow for the selective isolation and crystallization of the complexed species. We can selectively induce sedimentation for the heaviest molecular species. Because the complexed molecules are subtracted from the bulk solution, the reaction proceeds further toward the formation of complexes.

During the time course of a solution NMR experiment, molecules tumble and sample all different orientations at a rate faster than the difference in resonance frequencies among the different orientations. Therefore, each set of equivalent nuclei produces a single NMR signal at the average frequency. The rate of tumbling of a macromolecule in solution is dictated by its size but also by the surrounding environment. If fast interconversion between the different orientations is impeded (e.g., by other neighboring molecules), all the different frequencies are observed at once, giving rise to what is called a "powder pattern". This is the situation of solids, like microcrystalline samples, frozen solutions, or lyophilized samples (or otherwise immobilized molecules). In these cases, coherent averaging by mechanical rotation (magic angle spinning, MAS) can supplement the lack of incoherent averaging from the tumbling.[1]

In addition to crystallization, freezing, and lyophilization, samples of immobilized macromolecules amenable for solid-state NMR (SS-NMR) studies can be produced by sedimentation.[2] In this Account, we present and discuss the advantages and opportunities offered by SS-NMR of sedimented solutes (SedNMR), along with practical suggestions for experiment optimization.

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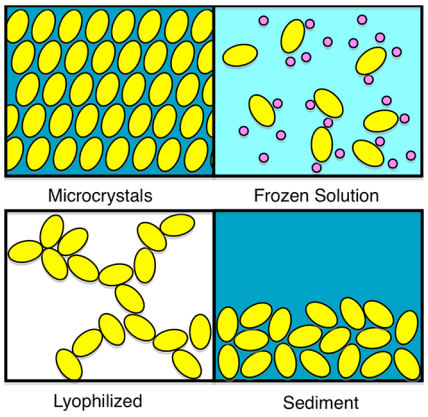

FIGURE 1. Different techniques of sample preparation for SS-NMR experiments: crystallization, freezing with cryoprotectants, lyophilization, and sedimentation.

Crystallization, Freezing, and Lyophilization: Sample Preparation in Solid-State NMR

Protein SS-NMR samples usually fall into one of these categories: microcrystalline samples, frozen solutions, or lyophilized powders (Figure 1). Pioneering protein SS-NMR studies relied on lyophilized samples;[3−5] frozen protein solutions were also used for biophysical characterization with minimal sample manipulation.[5,6] However, both samples usually yield poorly resolved spectra.[7,8] Frozen solutions can be easily studied at low temperatures, with the advantage of polarization enhancement.[9] Cryoprotectants[10] and lyoprotectants[7] are usually included in the preparation to avoid cold denaturation, although in some cases they may interfere with the normal protein activity. Frozen solutions in glycerol/water mixtures are the gold standard for sample preparation in solid-state dynamic nuclear polarization (DNP).[11]

The use of crystalline materials in SS-NMR dates back to the 1980s.[12] Micro- and nanocrystalline preparations, akin to those used in X-ray single-crystal or powder diffractometry, usually give rise to high-resolution spectra[13] and in the past decade have brought SS-NMR to compete with solution NMR in terms of experiments[14−16] and spectral quality.

Although both X-ray crystallography and SS-NMR rely on crystalline samples, they have different requirements: X-ray crystallography requires 20−30 μm crystals, usually soaked in glycerol and frozen at liquid nitrogen temperature to minimize radiation damage. Only rarely do systems behave well enough to allow for structural reconstruction from powder diffraction.[17] On the other hand, crystals for SS-NMR have no size requirements, as long as crystal order is preserved.[13] Such powder preparations are referred to as nanocrystals or "precipitates". Freezing is usually avoided because it deteriorates spectral quality. Another important issue is that the high concentrations of salts usually required in X-ray preparations increase RF heating in SS-NMR unless E-free probes are used.[18]

SS-NMR is the elected technique to study insoluble proteins, such as aggregates,[19] fibrils[20] and membrane proteins, which can be tackled by SS-NMR even in their native environment.[21]

Sedimentation

As an alternative way of producing samples of immobilized macromolecules, one may think of decreasing the protein rotational diffusion by adding small cosolutes, which increase the viscosity of the solution according to the Stokes−Einstein equation. However, it can be calculated that even particles as large as hundreds of kilodaltons in highly viscous glycerol−water mixtures have reorientation times on the order of microseconds. This is still too fast to consider the protein immobilized on the NMR time scale, because the spreading of the nuclear interaction frequencies is in the kilohertz range.

Increasing the concentration of the macromolecular solute increases only the macroscopic viscosity but not the microscopic viscosity; increasing the macromolecular concentration would thus not seem to be a viable route to slow down rotation. However, this is true only until the fractional volume occupied by the solute is negligibly small compared with the volume of the solution. When high concentrations of macromolecules are involved, a large nonlinear increase in the microscopic viscosity, due to molecular crowding, and thus a reduction of the rotational diffusion are expected and observed, depending on the nature of the macromolecules.[22]

An easy way to increase the concentration up to very high values is using gravity for achieving sedimentation. A macromolecular sediment can be considered to be an extremely concentrated solution.[23,24] The maximum possible concentration depends on the nature of the macromolecules.[24] In the case of globular proteins, concentrations of the order of 700 mg/mL are attained,[24] and molecules occupy about 60% of the sample volume. The concomitant reduction of translational diffusion makes the sample appear as a transparent and sticky glass.

Self-crowding is thus expected to increase sizably the protein reorientation correlation times, up to the point that the molecule can be considered immobile on the NMR time

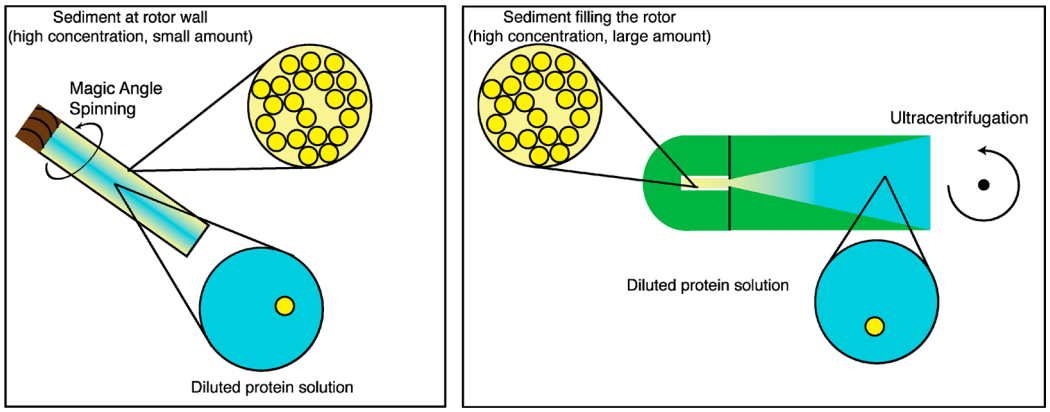

FIGURE 2. Summary of the approaches to achieve sedimentation for SS-NMR sample preparation: (left) *in situ* sedimentation; (right) *ex situ* sedimentation using ultracentrifugal devices.

scale. Even if rotational diffusion is hampered, hydration is likely to be largely preserved, and measurements can be performed at any temperature between the freezing of the bulk solvent and the thermal disruption of the protein. In this sense, sedimentation is an ideally suited approach for SS-NMR sample preparation.

Recent studies have highlighted that high concentrations can exert varied effects on protein fold and stability[25,26] and thus, at the high concentrations achieved during sedimentation, different proteins can respond in different ways. *E. coli* cells have been shown to survive and even proliferate at the high gravity achieved in ultracentrifuges,[27] and living cells have been used for MAS SS-NMR.[28,29]

For the practical purpose of performing SS-NMR, sedimentation can be achieved in two ways (Figure 2): it can be induced via an ultracentrifuge (UC-induced sedimentation or *ex situ*), or it can be directly driven by the centrifugal force that is exerted during magic angle spinning itself (MAS-induced sedimentation or *in situ*).

***In Situ* Sedimentation: Sediment Formation.** The solution sample is sealed as such in the SS-NMR rotor and subjected to MAS. After some time, a sediment layer is formed, depending on the rotor radius, spinning rate, and the sedimentation coefficient of the protein.[30]

By adapting the sedimentation equilibrium equations[31] to the geometry of the MAS rotor, eq 1 is obtained[32] to calculate the protein concentration, $c(r)$, as a function of the distance from the rotation axis, r, and the empirically determined maximum achievable concentration, c_{limit},

$$c(r) = \frac{c_{limit}}{1 + A \exp\left[-\frac{M(1 - \rho_{solvent}/\rho_{protein})\omega_r^2 r^2}{2RT}\right]} \quad (1)$$

with the integration constant A given by

$$A = \frac{\exp\left[\frac{M(1 - \rho_{solvent}/\rho_{protein})\omega_r^2 b^2}{2RT}\left(1 - \frac{c_0}{c_{limit}}\right)\right] - 1}{1 - \exp\left[-\frac{M(1 - \rho_{solvent}/\rho_{protein})\omega_r^2 b^2}{2RT}\frac{c_0}{c_{limit}}\right]} \quad (2)$$

where b is the rotor radius (m), c_0 the protein concentration in the static solution (mol/dm^3), M the molecular weight of the protein (kg/mol, kDa), ω_r the angular speed of the rotor (rad/s), ρ the density (kg/dm^3), R the universal gas constant, and T the absolute temperature (K). From the concentration profile, the amount of sediment that forms at the rotor wall can be calculated. Figure 3 shows the concentration profiles for three proteins of different MW (cytochrome *c* (12 kDa), bovine serum albumin (BSA, 66 kDa), and αB-crystallin (600 kDa)) at different spinning rates in a Bruker 4 mm rotor at 274 K.

Equations 1 and 2 show that the success of the experiment depends on the rotor radius and on the maximum spinning rate attainable with the rotor. Table 1 reports the minimum molecular weight that is required to sediment a protein at 100 mg/mL concentration and the minimum concentration that is required to sediment a protein of 50 kDa for commercially available rotors.

The equations also cast a grim light onto the addition of small molecules, like glycerol and sucrose, which are common additives in the case of freezing and lyophilization, respectively, but have the effect of increasing the density of the solution, thereby increasing the $\rho_{solvent}/\rho_{protein}$ ratio to values closer to 1 (or even above 1). The concentration profiles for the same proteins analyzed in pure water are

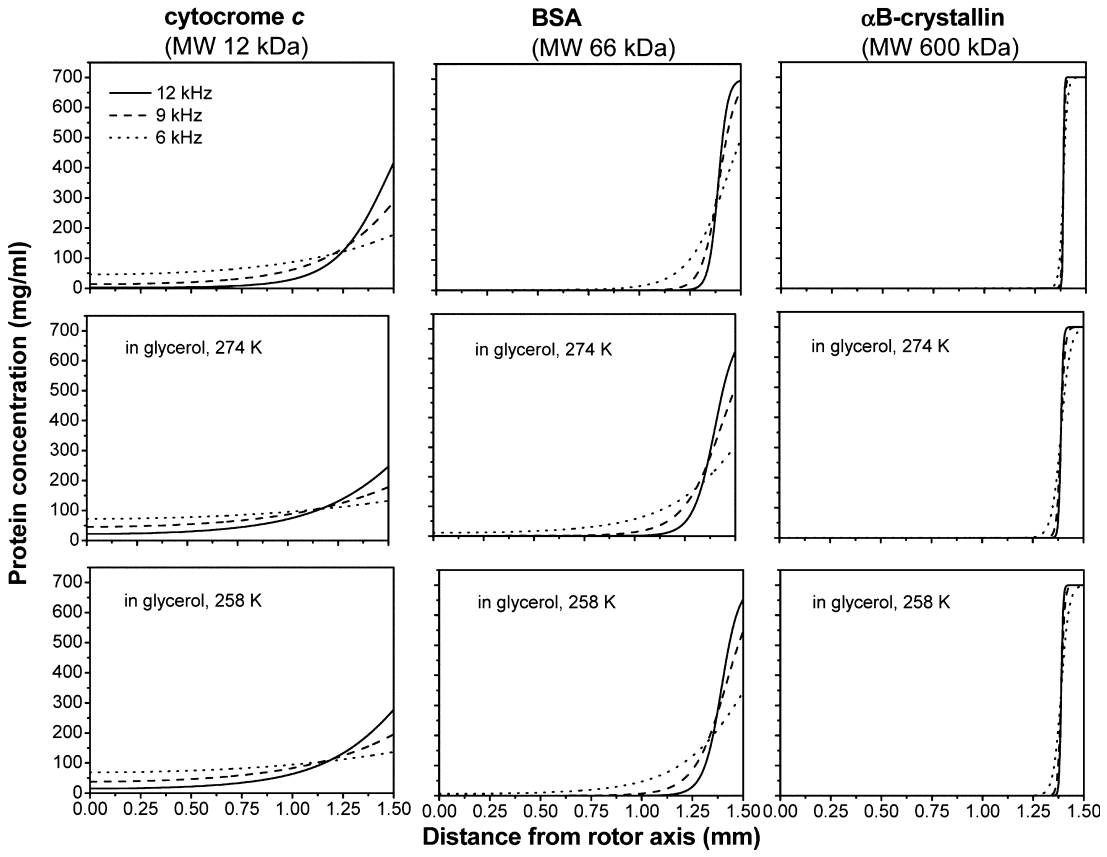

FIGURE 3. Concentration as a function of the distance from the rotor axis at different spinning rates (12, 9, and 6 kHz) for cytochrome *c*, BSA, and αB-crystallin and protein concentration, c_0, in the static solution of 100 mg/mL, in a 4 mm rotor (1.5 mm internal radius). The limiting concentration is set to 700 mg/mL. The concentration profiles are reported for water−protein solutions at 274 K, for solutions with 40% glycerol at 274 K, and for solutions with 40% glycerol at 258 K.

TABLE 1. The Minimum Molecular Weight Required To Sediment a Protein at 100 mg/mL Concentration in Solution and the Minimun Protein Concentration in Solution Required To Sediment a Protein of 50 kDa Calculated for the Commercially Available Bruker and Agilent Rotors

rotor	interior diam (mm)	max speed (Hz)	min MW (kDa)	min. concn (mg/mL)
Agilent, 1.2 mm, std.	0.625	60000	235	470
Bruker, MAS 1.3	0.9	67000	91	181
Agilent, 1.6 mm, std.	1.143	40000	157	331
Bruker, MAS 1.9	1.5	42000	83	164
Agilent, 2.5 std	1.5748	30000	145	295
Bruker, MAS 2.5	1.7	35000	93	186
Agilent, 3.2 mm, std.	2.032	25000	126	251
Agilent, 4.0 mm, std.	2.4638	18000	164	333
Bruker, MAS 3.2	2.6	24000	85	167
Agilent, 3.2 mm, thin wall	2.6162	15000	212	426
Bruker, MAS 4	3	15000	161	322
Agilent, 4.0 mm, thin wall	3.2258	10000	313	606
Agilent, 5.0 mm, std.	3.429	12000	192	388
Agilent, 5 mm, thin wall	4.3942	7000	344	633
Agilent, 6.0 mm, std.	4.4958	9000	200	399
Bruker, MAS 7	5.6	7000	212	426
Agilent, 7.5 mm, std.	5.969	7000	188	371
Agilent, 9.5 mm, std.	7.9502	5500	172	345

shown in the presence of 40% glycerol in Figure 3, in the same rotor and at 274 K. The increased density *decreases* the concentration gradient that is attainable at equilibrium. Even at 258 K (before the phase transition of the glycerol/water mixture), sedimentation remains less efficient than in the absence of glycerol at 274 K. Moreover, the reduction in the

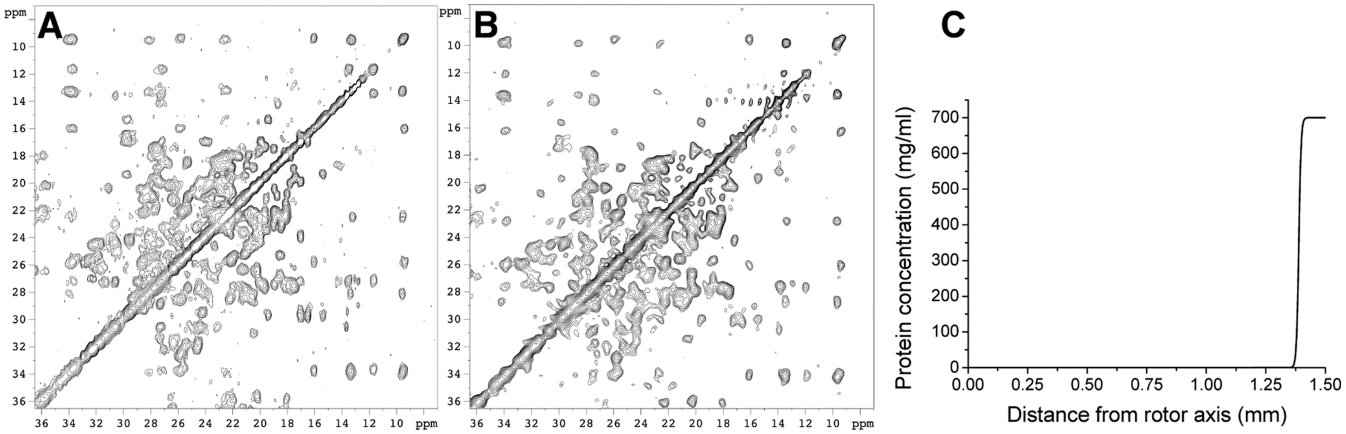

FIGURE 4. Aliphatic portions of 2D DARR spectra of microcrystalline (A) or sedimented (B) apoferritin, recorded at 298 K, 9 kHz MAS, and 100 kHz ^{1}H decoupling. (C) Corresponding concentration profile for the solution of 0.125 mM apoferritin.

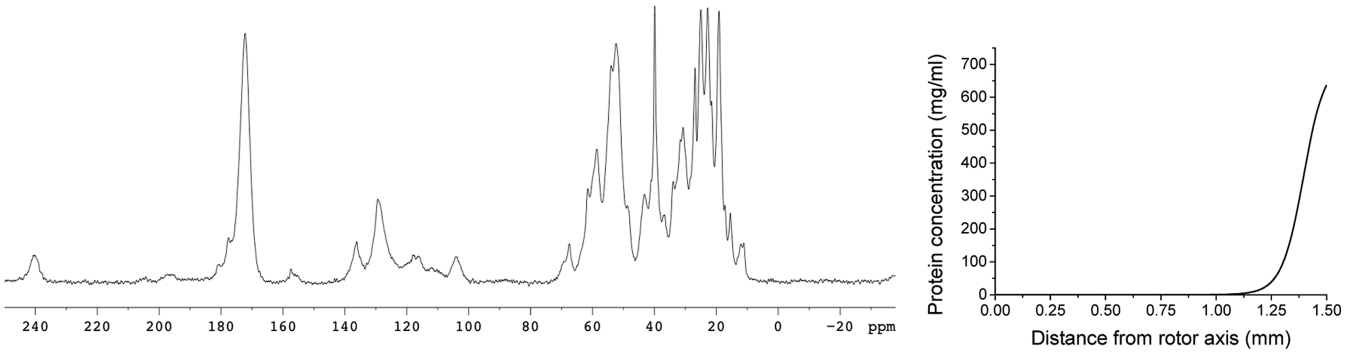

FIGURE 5. ^{1}H−^{13}C cross-polarized spectrum of ^{13}C-enriched carbonic anhydrase II (100 mg/mL, 100 kHz decoupling, 12 kHz MAS, 16.5 T, 277 K) and the corresponding concentration profile of the protein.

dielectric constant of the medium by the addition of an organic molecule in the aqueous solution increases the repulsion between pairs of like molecules[33,34] up to the point that eventually the protein molecules in the sediment will be less prone to come close to one another and slow down their rotational motions.

It can also be noted that the equilibrium concentration distribution is not achieved instantly, and the required time depends on the viscosity of the solution: the use of a glycerol/water mixture increases the viscosity of the solution and thus decreases the sedimentation velocity.

***In Situ* Sedimentation: Experiments.** We have shown that it is possible to obtain high-resolution SS-NMR spectra on a sedimented sample of the 24 × 20 kDa protein apoferritin, with NMR peaks even sharper than for the corresponding microcrystalline preparation under the same experimental conditions (Figure 4A,B).[2] The concentration gradient that is formed during MAS is reported in Figure 4C, with a protein concentration of 0.125 mM (corresponding to a concentration of 3 mM of the monomer). Sedimentation was shown to occur even for a protein as small as carbonic anhydrase II (29 kDa, see Figure 5).[32] The concentration gradient achieved under the experimental conditions is also reported in the figure. In this case, only a fraction of the protein can be sedimented.

In situ sedimentation was applied for the study of the interaction of copper ions with the αB-crystallin protein,[35] a polydisperse ensemble of 10−40 identical 20-kDa subunits,[37] resulting in molecular masses ranging from 200 to 800 kDa. The large molecular weight ensures sedimentation during MAS, and the acquired spectra show NMR lines narrow enough to allow for site-specific monitoring of the metal−protein interactions by the disappearance of resonances due to paramagnetic relaxation.

A protein may have rigid and mobile segments. If mobile parts are preserved in the solid state, they respond to *J*-based experiments (INEPT),[36] while the more rigid parts respond to cross-polarization (CP).[37] It has been shown that comparison between the spectra acquired under the two different excitation schemes can permit one to determine the presence

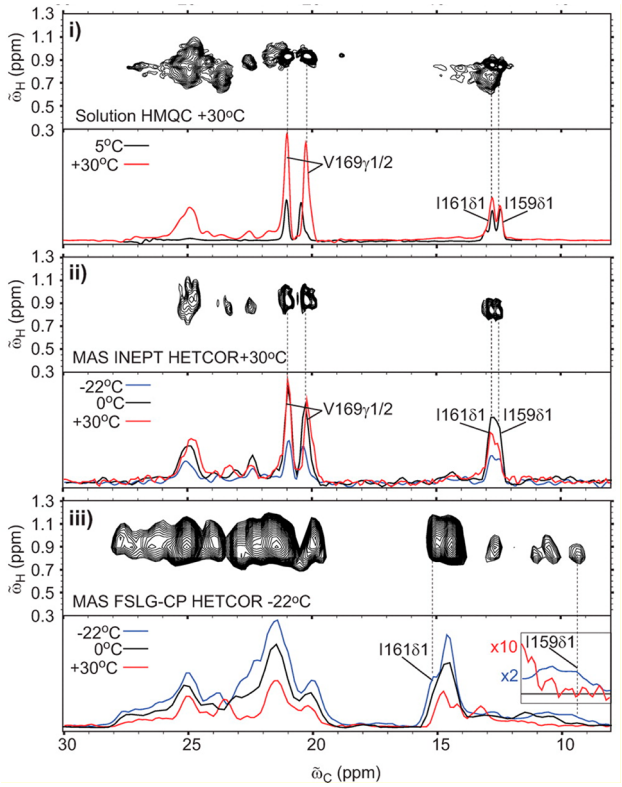

FIGURE 6. Methyl region of the $^{1}H-^{13}C$ NMR spectra obtained for U-[^{2}H], Ile-[$^{13}CH_3$ δ1], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] αB-crystallin in solution (i), in a sedimented sample with ^{1}H to ^{13}C polarization transfer achieved via INEPT to highlight the dynamic parts (ii), and in a sedimented sample with polarization transfer achieved via CP (iii). The spectrum shown in part ii is very similar to that in part i. The resonances from I159δ1 and I161δ1 present in part iii at 9.5 and 15 ppm, respectively, clearly visible at −22 °C are no longer observed at temperatures of 0 °C and higher. Reproduced with permission from Baldwin et al.[38] Copyright 2012 American Chemical Society.

of flexibility in some protein regions. This approach has allowed researchers to study the conformational dynamics at the oligomer interface in αB-crystallin[38] (see Figure 6).

***In Situ* Sedimentation: Advantages.** *In situ* sedimentation allows for the study of large objects that exceed the size for satisfactory characterization by solution-state NMR, comprising biologically relevant targets such as complexes and oligomeric aggregates.[39] With respect to freezing and lyophilization, the resolution is usually better, since the protein remains always in a largely hydrated state.

Although protein crystallization has become routine, it may still happen that no crystals at all are obtained, especially in the case of highly charged proteins. In principle, NMR lines of sediments are narrower than those of the corresponding microcrystalline preparations.[32] Finally, and possibly more importantly, when MAS is stopped, the protein tends to diffuse back from the sediment to the bulk

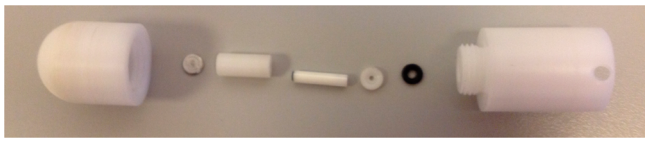

FIGURE 7. Components of the ultracentrifugal device designed for packing sedimented solutes directly into the SS-NMR rotor.[42]

solution, in a time that depends on the translational diffusion coefficient of the protein, reverting to the initial buffer and concentration conditions. This allows for the kind of sample handling that is common for solution-state NMR.[35]

As common for analytical ultracentrifugation,[31] since sedimentation achieved directly in a spinning MAS rotor inside the NMR magnet can take a considerable amount of time to complete, spinning can be performed to the maximum rate to facilitate the onset of the equilibrium, and then decreased to match the desired one ("overspeeding" approach).

***Ex Situ* Sedimentation: Sediment Formation.** *Ex situ* sedimentation is achieved via ultracentrifugation. This strategy allows for sedimentation of smaller proteins, and it is more efficient in terms of amount of sample in the MAS rotor. In fact, preparation of protein solutions at concentrations higher than 400 mg/mL is unpractical, while c_{limit} values of about 700 mg/mL are attained during ultracentrifugation of solutions at moderate to high concentration: the rotor filled *ex situ* thus easily contains at least a factor 2 more sample.

This is especially important in the case of the 1.3 mm rotor, which permits one to obtain spectra with the highest resolution but has a small internal volume (∼2 μL). The increase in sensitivity that can be achieved on passing from *in situ* to *ex situ* sedimentation can be dramatic.[32]

The preparative ultracentrifuge tubes are already well-suited for sample collection, although one must pay great attention to the possibility that the protein will diffuse back into the solution in the time course between ultracentrifuge braking and sample collection. The time required to complete sedimentation according to the Svedberg equation[40] will dictate the success of the experiment.

We have proposed the use of ultracentrifugal devices (Figure 7), like the one described by Bockmann et al.[41] for packing microcrystals, to form and funnel the macromolecular sediment directly into the SS-NMR rotor as a tool to further improve the sample preparation.[32,42] This approach was later applied by Gardiennet et al.[43] to obtain high-resolution spectra of a dodecameric helicase, a protein of 12 × 59 kDa; also in this case the resolution of the sediment

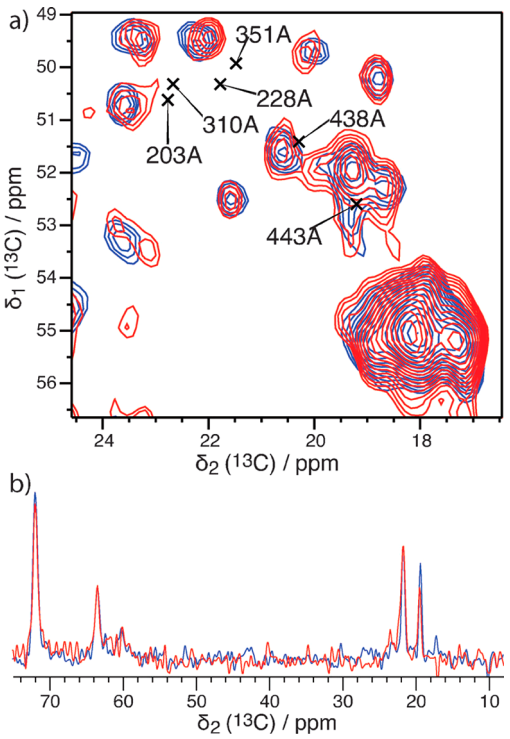

FIGURE 8. Alanine region of the 2D DARR spectra of the dodecameric helicase DnaB (sedimented sample, red; microcrystalline sample, blue). Crosses indicate SHIFTX predictions. (b) One-dimensional traces through the same spectra at $\delta_1 = 71.9$ ppm. Reproduced with permission from ref 43. Copyright 2012 Wiley.

is comparable to that of the microcrystalline preparation, with peak positions in good agreement with those observed in the microcrystalline preparation (see Figure 8).[43] The minimum molecular weight to sediment a protein from a solution at 30 mg/mL is about 9 kDa using the device described in ref 42.

***Ex Situ* Sedimentation: Advantages.** *Ex situ* sedimentation has the obvious advantage of using a rotor completely filled with the sediment, with respect to the *in situ* sedimentation, as already noted above. The amount of sample in the rotor is comparable to that of a microcrystalline preparation: a volume of about 50−60%[44] is in fact usually occupied by proteins in crystals, corresponding to concentrations of about 600−700 mg/mL.

A Sediment-Based Overview of Previous Literature

The approach of hydrating a lyophilized protein just up to the point where the protein is barely redissolved has been employed for lysozyme,[45] BSA,[46] α-spectrin SH3,[7] and ubiquitin.[47] As long as a solid component is present, SS-NMR spectra are expected to be detectable. In the case of ubiquitin (6 mg) hydrated with H_2O (10 μL) after lyophilization,[47] the concentration of the protein is around 400 mg/mL. Under the reported experimental conditions,[47] the concentration gradient in Figure 9A is obtained. The high quality of the NMR spectra strongly suggests that a rehydrated lyophilized material corresponds directly either to a sediment or to an extremely concentrated solution that easily forms a sediment under MAS. SS-NMR spectra acquired for BSA after hydration of a lyophilized sample also show sharp resonances,[46] comparable to those observed for the sedimented protein,[32] suggesting that the sample is in the same physical state in the two cases.

In situ sedimentation is also likely at the origin of the observation of solid-state spectra of αB-crystallin in Mainz et al.[48] Under the reported experimental conditions, in fact, the concentration gradient shown in Figure 9B is formed, so the whole protein is expected to be sedimented.

Sedimentation Driven by Binding Equilibria

Proteins often perform their biological actions in complexes having too large a MW for solution NMR studies and too low a binding constant for selective isolation and crystallization of the complexed species. *In situ* SedNMR can be a simple way to access the NMR signals of the species of interest, it being either stable or fluxional. In fact, under centrifugation in the MAS rotor, the complexed molecules are preferentially subtracted from the bulk solution and undergo sedimentation, so that reactions are further shifted toward the formation of sedimented complexes.

We have used the treatment by Chatelier and Minton[49,50] for the study of *in situ* sedimentation in the presence of binding reactions at chemical equilibrium (i.e., both sedimentation and reactions are at equilibrium in any point of the rotor). The effect of macromolecular crowding on the kinetics of aggregation and dissolution is not considered, and the complex is assumed able to dissociate at any concentration, although we might expect that once the formed complex has been dragged into the sediment layer, it will be less prone to dissociate into the individual molecules.

A series of thought experiments shows that it is indeed possible to select by sedimentation the heaviest molecular species even when aggregation is disfavored. This holds even more when dissociation of the complex in the sediment becomes too slow to be efficient.

Figures 10 and 11 show the results of numerical simulations of sedimentation experiments in a 4 mm Bruker rotor (see Table 1) of (a) a 25 kDa protein with concentration 100 mg/mL, aggregating into dimers, trimers, and tetramers

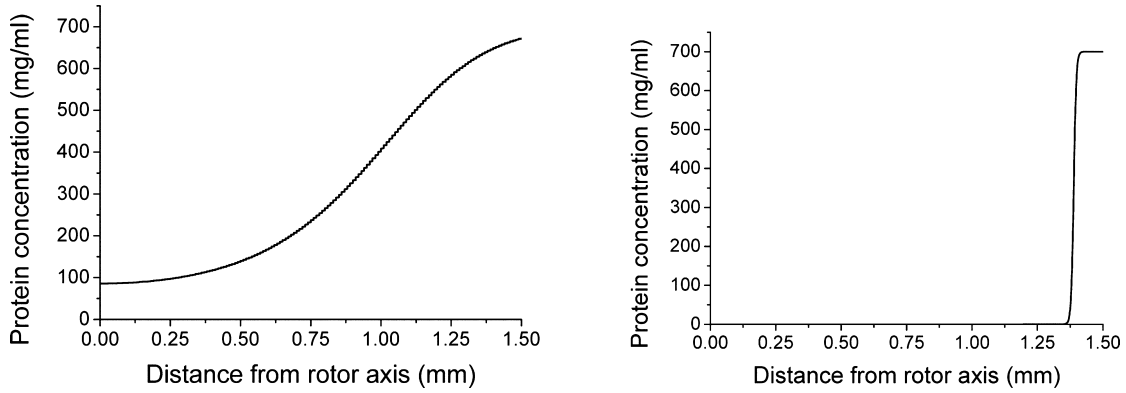

FIGURE 9. Concentration as a function of the distance from the rotor axis for (A) ubiquitin (conditions, MAS = 11 kHz, concentration in the static solution of 400 mg/mL)[47] and (B) αB-crystallin (conditions, MAS = 12 kHz, concentration in the static solution of 100 mg/mL, $T = 263$ K).[48]

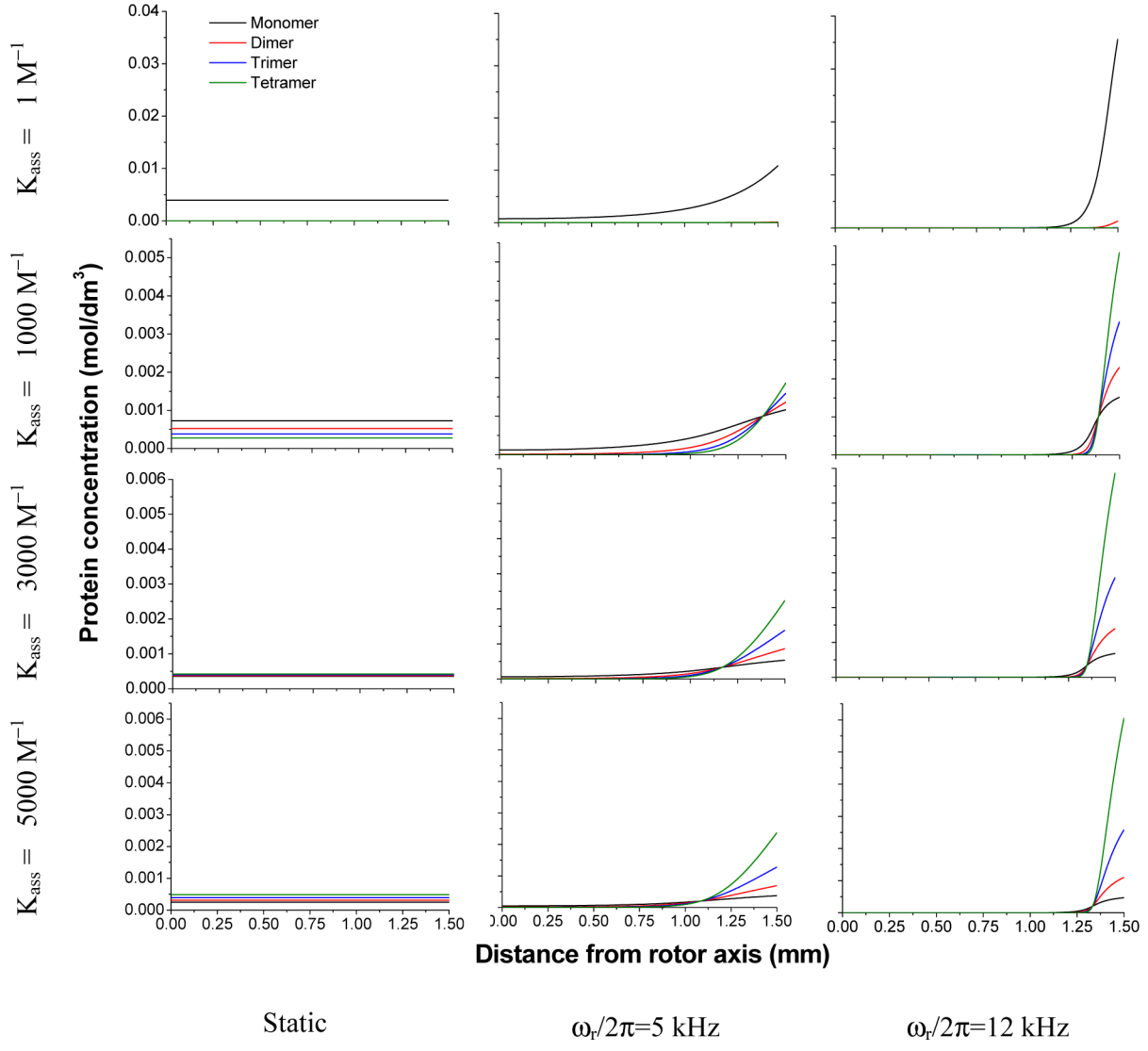

FIGURE 10. Concentration as a function of the distance from the rotor axis in the case of homoaggregation with association constants set equal to 1, 1000, 3000, or 5000 M^{-1}.[49]

through the sequential reactions $A + A = A_2$, $A + A_2 = A_3$, $A + A_3 = A_4$, with binding constants $K_1 = K_2 = K_3$, all equal and amounting to either 1, 1000, 3000, or 5000 M^{-1} (Figure 10) and (b) a 20 kDa protein (A) at 1 mM concentration,

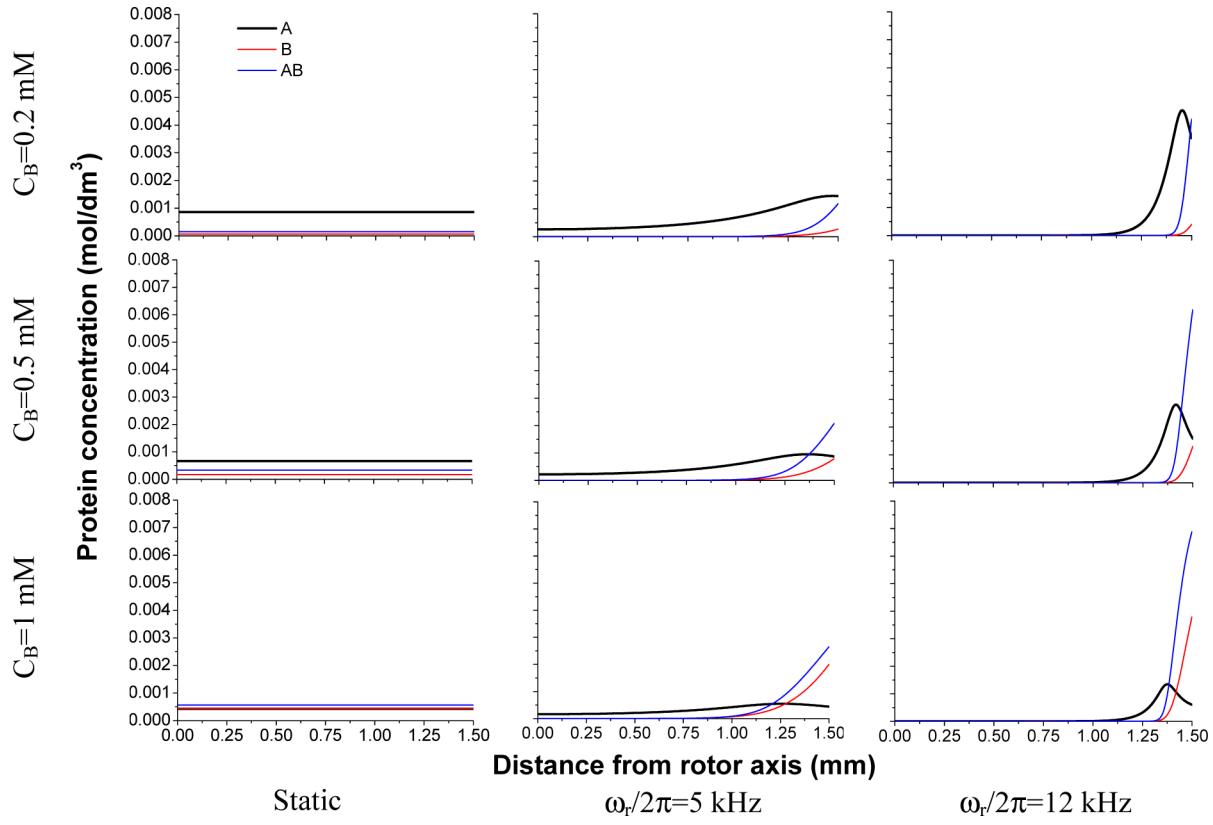

FIGURE 11. Concentration profiles as a function of the distance from the rotation axis of a two component system A + B forming the complex AB with $K_{eq} = 3000\ M^{-1}$. The concentration of the species A (20 kDa) is 1 mM. The concentration of the species B (80 kDa) is indicated.[50]

interacting with an 80 kDa protein (B) at concentrations ranging from 0.2 to 1 mM to form a complex (AB) with a binding constant of 3000 M^{-1} (Figure 11).

The results suggest that there are cases where, although polimerization is not strongly favored in the static solution, the most aggregated species may prevail in the sediment and can thus be detected and studied by SS-NMR. This is the case of the tetramerization shown in Figure 10, with binding constant of 1000−5000 M^{-1}.

It is also interesting to analyze the case of two hetero-associating proteins. By solution NMR, the study of this system requires different experiments depending on the kinetics of association/dissociation and perhaps requires perdeuteration to cope with the large size of the complex. Sedimentation can be used to enrich the sample with the desired component, depending on the relative concentrations of the reactants. As shown in Figure 11, when the two proteins A and B are in a 1:1 ratio at 12 kHz, the sediment is almost completely devoid of free A. Therefore, if the lighter protein A is labeled, only the complexed form of A will be visible through SS-NMR experiments. This is at variance with the static solution, where almost equal amounts of A and AB are present. On the other hand, the detection of the complexed form of the heavier labeled protein B would require maximization of the amount of the bound form of B in the sediment with respect to the free form. Intuitively this can be done by increasing the A/B ratio to, say, 1:0.2. A slight drawback is that about 20% (in mass) of the sediment is composed by the unlabeled protein A, with a concomitant modest lowering of the signal-to-noise ratio. In the intermediate situation of 1:0.5 in the A/B ratio, the concentrations of both free A and free B are lower than the concentration of the complex in the sediment. This ratio is thus apparently favorable for detection of both A and B in the complexed form. To be noted, the decrease in the concentration of the protein A by approaching the walls of the rotor is due to the crowding effect caused by the sedimentation of the larger MW species B and AB.

Conclusions

The SedNMR approach, initially demonstrated by Bertini et al.[2] by the detection of solid-state NMR spectra of a water solution of apoferritin under MAS, promises to be a precious tool for the study of biomolecules, which can be hardly studied by solution NMR or SS-NMR. It can allow investigation of large molecules, transient systems, and adducts with

a low binding constant not detectable in static conditions. Recently, it was shown that, upon freezing, the sediment can form a glass suitable for dispersing biradicals[51] and achieving DNP.[52]

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BIOGRAPHICAL INFORMATION

Ivano Bertini, born on December 6, 1940, in Pisa, Italy, obtained the Italian degree of Doctor of Chemistry at the University of Florence in 1964 and became Full Professor of General and Inorganic Chemistry in 1975 at the University of Florence. Member of the Academia Europaea and the Italian Accademia dei Lincei, he received the Laurea Honoris Causa from the University of Stockholm, Ioannina, and Siena. In 1999, he founded the Magnetic Resonance Center (CERM), a major NMR infrastructure in the Life Sciences. He published over 650 research articles and solved more than 150 protein structures. He passed away on July 7th, 2012.

Claudio Luchinat, born in Florence, Italy, on February 15, 1952, obtained his doctor in chemistry cum laude at the University of Florence, was a postdoctoral associate and researcher at the University of Florence, and Full Professor of Chemistry at the University of Bologna (1986−1996) and Florence (1996 to the present). He is a recipient of the "Raffaello Nasini" gold medal award for inorganic chemistry of the Italian Chemical Society, 1989; Federchimica Award "For an Intelligent Future", 1994; European Medal for Biological Inorganic Chemistry, 1996; and "GDRM gold medal for magnetic resonance", 2001. He is the author of almost 600 publications in bioinorganic chemistry, NMR, structural biology, and biophysics, and of four books.

Giacomo Parigi, born in Borgo San Lorenzo, Italy, on September 17, 1967, graduated in physics and obtained his Ph.D. in chemistry at the University of Florence, Italy. He was postdoctoral associate and researcher and is Associate Professor of Chemistry since 2006 at the University of Florence. His research interests include NMR effects related to paramagnetism for the structural and dynamic characterization of biomolecules and to nuclear and electron relaxation.

Enrico Ravera, born in Arezzo, Italy, on April 1, 1986, obtained a B.Sc. in Chemistry in 2008 and M.Sc. in Chemistry cum laude in 2009. He is currently a postdoctoral fellow under the supervision of Prof. Luchinat.

FOOTNOTES

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The authors declare no competing financial interest.

We dedicate this work to Ivano Bertini and recall with fondness the many brainstorming sessions that we have had with him on SedNMR, a discovery of which he was particularly proud.

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