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Heat Management Strategies for Solid-state NMR of Functional Proteins

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Abstract

Modern solid-state NMR methods can acquire high-resolution protein spectra for structure determination. However, these methods use rapid sample spinning and intense decoupling fields that can heat and denature the protein being studied. Here we present a strategy to avoid destroying valuable samples. We advocate first creating a sacrificial sample, which contains unlabeled protein (or no protein) in buffer conditions similar to the intended sample. This sample is then doped with the chemical shift thermometer $\text{Sm}_2\text{Sn}_2\text{O}_7$. We introduce a pulse scheme called TCUP (for Temperature Calibration Under Pulseload) that can characterize the heating of this sacrificial sample rapidly, under a variety of experimental conditions, and with high temporal resolution. Sample heating is discussed with respect to different instrumental variables such as spinning speed, decoupling strength and duration, and cooling gas flow rate. The effects of different sample preparation variables are also discussed, including ionic strength, the inclusion of cryoprotectants, and the physical state of the sample (i.e. liquid, solid, or slurry). Lastly, we discuss probe detuning as a measure of sample thawing that does not require retuning the probe or using chemical shift thermometer compounds. Use of detuning tests and chemical shift thermometers with representative sample conditions makes it possible to maximize the efficiency of the NMR experiment while retaining a functional sample.

Keywords

Temperature calibration; TCUP; sample preservation; ionic strength; cryoprotectant; sacrificial sample; chemical shift thermometer

1. Introduction

Advances in solid-state NMR methodologies have made it possible to measure local or complete structure of proteins in important noncrystalline states, such as membrane proteins in lipid bilayers,[1] viral coat proteins in intact phage particles,[2] and amyloid-forming proteins in fibrils.[3, 4] For successful structure/function analysis by solid-state NMR,

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at ...

protein samples must be protected from excessive heating by the NMR experimental conditions such as high magic angle spinning speeds [5–7] and high-power radiofrequency (RF) pulses.[6, 8, 9] Heating can be sizable: sample temperature increases of over 30°C have been demonstrated due to proton decoupling pulses in samples containing hydrated lipid bilayers[6] or high ionic strength conditions[9] or due to ultrafast (35 kHz) magic angle spinning speeds.[10] Although new probe designs are available that minimize sample heating by the pulses,[8, 9, 11–16] it is critical to verify that the temperature will remain within an acceptable range for a given sample and NMR experiment.

The degree of heating depends on a number of parameters including the spinning speed, temperature and volume of the cooling and spinning gases, power, duration, and repetition rate of the pulses, and the composition of the sample itself. Heating can be reduced by decreasing the spinning speed, pulse power, or repetition rate, but each of these changes can also reduce the quality of the NMR data. Here we demonstrate an approach to minimize the risk of damaging a valuable sample while maximizing the efficiency of the NMR experiment: heating of a representative sample subjected to the NMR experimental conditions is measured and used to guide refinement of the sample and NMR conditions as needed.

The majority of the chemical shift thermometers that have been described are water-soluble ionic compounds (KBr, $\text{PbNO}_3\text{SmAc}_3\text{TmDOTP}$).[6, 17–19] Such compounds will typically dissolve if added to the solid state NMR sample of interest (in our case an aqueous slurry of buffer, lipids, and protein), which may perturb the heating properties of the sample through two effects. First, it will increase the ionic strength of the sample, which increases the degree to which the sample is heated by radiofrequency electrical fields.[9] Second, it will lower the melting point of the sample through colligative effects. This may also substantially change the rate at which the sample absorbs heat, as RF-induced heating of liquid aqueous salt solutions is much greater than that of solid ones. These effects can be avoided if the compound is kept separate from the sample, as in a recent report that measured temperatures using a small plug of KBr powder.[6] However, if there are significant thermal gradients or limitations in heat transfer, such an external standard may not accurately reflect the temperature throughout the sample. We chose to use $\text{Sm}_2\text{Sn}_2\text{O}_7$ [20] as an internal chemical shift thermometer, because its insolubility in aqueous solution makes it possible to measure temperature within the sample without altering the heat absorption and freezing properties of the sample.

Productive solid-state NMR studies can be conducted on either frozen or unfrozen states of functional proteins, depending on the goals and constraints of the study. Static NMR measurements of orientations are typically performed on unfrozen bicelle or glass plate samples to achieve good alignment. For magic angle spinning studies, motional averaging in unfrozen proteins sometimes decreases signal intensities and linewidths. The latter is critical for maximizing resolution in spectra of uniformly or extensively labeled proteins, so unfrozen samples are often used for multidimensional NMR studies seeking extensive assignments and structure determination. In contrast, selected distance measurements are often conducted on frozen proteins, to avoid motional averaging of the dipolar couplings being measured. Freezing the sample can minimize heat absorption under high ionic strength conditions that may be required for functionality, as demonstrated below, but may also cause freeze/thaw damage.

Here we report a successful strategy that uses $\text{Sm}_2\text{Sn}_2\text{O}_7$ to test the desired sample and NMR conditions to determine the magnitude of the sample heating and whether the sample temperature stays well below the freezing point during pulsing. Such measurements may be critical to maintaining functional samples in NMR of unfrozen and frozen proteins,

respectively. Since complete freezing of a complex sample is not always readily apparent or predictable, simple tests for sample thawing during the NMR experiment are also described. These approaches provide the opportunity to address any problems with heating or thawing via adjustments to the sample conditions (eg ionic strength and choice of cryoprotectant) or the NMR conditions (eg spinning rate, cooling gas flow rate, pulse power and duty cycle). These adjustments can all be made before risking damage to a valuable protein sample. Finally, the rapid nature of the temperature measurement using $\text{Sm}_2\text{Sn}_2\text{O}_7$ provides insight into the best strategy for further minimizing sample heating. Use of these tests to optimize sample and NMR conditions enables NMR studies of functional proteins.

2. Results

2.1 $\text{Sm}_2\text{Sn}_2\text{O}_7$ shift measurements establish baseline sample temperatures in MAS experiments

Chemical shift measurements on a sample of $\text{Sm}_2\text{Sn}_2\text{O}_7$ over the temperature range of interest were used to establish an empirical relationship between sample temperature (T_s) and cooling gas temperature (T_g) for our instrumental conditions. The ^{119}Sn spectra at 5 kHz MAS speed were obtained with a simple 90° -observe experiment, which uses a minimum of power and is expected to introduce little or no heating from electromagnetic radiation. The measured chemical shifts were converted to temperatures (K) using the equation previously determined by Grey et al. for this compound in our temperature range [20]:

$$\delta = 223 - \frac{9.53 \times 10^4}{T} \quad (1)$$

As shown in Figure 1, these measurements (black triangles) establish a linear relationship (dashed black line) between the sample and the cooling gas temperatures for our instrumentation under these experimental conditions (5 kHz MAS, cooling gas flow rate of 100 scfh, no decoupling).

One reason for the temperature difference between the sample and the cooling gas is that room temperature gases (the bearing and drive gases in our probe configuration) also impinge on the sample. This predicts that the sample temperature will deviate towards room temperature, as observed: T_s and T_g are closest near room temperature and the slope less than one brings T_s towards room temperature over the whole range. The remaining temperature offset when the cooling gas is at room temperature is likely due to frictional heating by MAS. When $T_g = 298\text{K}$, $T_s = 299.7\text{K}$ so the frictional heating is only $\sim 2^\circ\text{C}$ for 5 kHz MAS under our conditions. This is consistent with what others have found at similar spinning speeds with similar-sized rotors.[6] Greater frictional heating is expected for larger rotors (the larger the rotor diameter, the greater the velocity at its edge for a given speed) and for higher spinning speeds. The observed small temperature increase also supports the claim that the 90° -observe pulse sequence does not significantly heat the sample. An empirical temperature calibration as shown in Fig. 1 depends on multiple parameters that include the rotor size, MAS speed (which affects both friction and the flow rate of room temperature gases), and the flow rate of the cooling gas.

Data obtained on the $\text{Sm}_2\text{Sn}_2\text{O}_7$ sample in another probe (gray inverted triangles) establish a very similar baseline, with a minor deviation at lower temperature (below -40°C). There is no significant change in this baseline for a sample containing DMPC vesicles in 150 mM KCl with 20 mg $\text{Sm}_2\text{Sn}_2\text{O}_7$ (circles), demonstrating that the presence of salt and lipid does not significantly alter the sample's heating properties under MAS alone.

2.2 Maintaining fully frozen samples is key to reducing RF pulse heating of high ionic strength samples

The other significant potential source of sample heating besides MAS is the electric field generated in the sample coil of the probe. The NMR experiment uses strong radiofrequency magnetic fields to manipulate nuclear spins, which are typically accompanied by substantial electric fields that can accelerate ions and dipoles in the sample and so lead to heating. An accurate prediction of the sample heating for an experiment of interest can be obtained by doping $\text{Sm}_2\text{Sn}_2\text{O}_7$ into a sample of similar composition and applying a pulsing scheme similar to the planned experiment.

Any pulse sequence of interest can be modified by replacing all the pulses on the observe channel with a series of closely-spaced 90-observe experiments on the ^{119}Sn channel. We refer to this strategy as TCUP (for Temperature Calibration Under Pulse-load); the example of a TCUP-modified ^{13}C - ^{19}F REDOR experiment is shown in Figure 2. The rapidly repeated observations are possible because the paramagnetic $\text{Sm}_2\text{Sn}_2\text{O}_7$ has a very short T_1 (measured here at 1.8 ms for the pure compound, and somewhat shorter in a high salt lipid sample; previously reported to be ≈ 0.1 ms [20]). Pulsing this way allows signal to be acquired more quickly, cutting down on the experiment time. It also allows the sample temperature to be followed with a high degree of time resolution, as discussed further below.

Many functional states of biological samples, including the protein complex of interest in our work,[21] require high ionic strength conditions (comparable to physiological ionic strengths), which have been shown to lead to substantial ($>30^\circ\text{C}$) sample heating during RF pulsing.[9] Such heating should be greater in a liquid than in a frozen sample: the greater mobility of ions in liquid solutions allows them to respond more strongly to oscillating electric fields and so absorb more heat. To test whether heating is tolerable in a frozen sample at the ionic strengths needed for our work, we performed TCUP-REDOR on 150 mM KCl solutions containing $\text{Sm}_2\text{Sn}_2\text{O}_7$. TCUP-REDOR experiments were conducted with a 3% duty cycle and 100 kHz ^1H channel power levels, using the pulse sequence shown in Figure 2 with $X=64$ and $n=56$. The 3 ms ^{119}Sn acquisition time plus 100 μs between acquisitions of ^{119}Sn spectra was sufficient for nearly complete ($\approx 80\%$) T_1 relaxation. Figure 3 shows a comparison of ^{119}Sn spectra obtained with a 90°-observe vs TCUP-REDOR pulse sequence, for temperatures at which the sample is unfrozen (A, initially at $T_s = 279\text{K}$) and frozen (B, initially at $T_s = 235\text{K}$). As expected, the unfrozen 150 mM KCl sample (A) shows a significant temperature increase: the chemical shift change indicates pulse-induced heating of about 11 K. Comparable heating of $11 \pm 1\text{K}$ was also observed for samples containing 50 or 100 mM KCl. In contrast, when the 150 mM KCl sample is cooled to -38°C (B) and fully frozen ($\text{H}_2\text{O}/\text{KCl}$ eutectic temperature = -10.7°C), the application of ^1H decoupling results in no detectable temperature change. Thus it should be possible to perform the REDOR experiment on a sample under high ionic strength conditions without damaging the sample, if it is kept fully frozen.

Note that a limitation of the $\text{Sm}_2\text{Sn}_2\text{O}_7$ chemical shift thermometer is that it may report on the temperature at the perimeter of the sample: since this insoluble compound has a high density of 7.5 g/cm³ MAS forces (particularly at high MAS frequencies) may drive it to the outer perimeter of the rotor. This tendency will depend in general on the particle size of $\text{Sm}_2\text{Sn}_2\text{O}_7$ used, the viscosity of the sample, and the duration and frequency of spinning. If the sample is to be frozen, the length of time it is spun while unfrozen is also a factor. For our experiments we dispersed finely ground $\text{Sm}_2\text{Sn}_2\text{O}_7$ in the sample, which was then spun briefly (~ 5 min at 5kHz) before being frozen. For lipid vesicle-containing samples (Fig 4 below) the sample was extruded from the rotor following the experiment and then sliced with a razor blade. Visual inspection of the cross-section suggested that $\text{Sm}_2\text{Sn}_2\text{O}_7$ remained uniformly distributed in this particular sample.

Since it is difficult to predict the freezing point in a complex biological sample containing salts, proteins, cryoprotectants, and other components, a prudent approach is to make an empirical measurement of heating on a simplified sacrificial sample of similar composition, before subjecting a more costly sample to the NMR experimental conditions. Our application to chemotaxis signaling complexes[21] requires a sample that contains 3 proteins, lipid vesicles, a high ionic strength buffer, and a cryoprotectant. A low-cost sacrificial sample composition for this case includes the lipid vesicles and only one of the three proteins, with no isotopic enrichment. These components were sedimented by centrifugation and mixed with $\text{Sm}_2\text{Sn}_2\text{O}_7$ to form the NMR sample. In systems where inclusion of even a single, un-enriched protein component is unduly costly, simply using the buffer of interest may be sufficient to estimate how much heating will occur in the eventual protein-containing sample.

Sample heating was compared for the lipid/protein complex in two buffer compositions that had been demonstrated to preserve significant protein activity after a slow freeze/thaw.[21] With cooling gas temperatures of -40°C , -50°C , and -60°C , the sample temperature was determined from the ^{119}Sn chemical shift of $\text{Sm}_2\text{Sn}_2\text{O}_7$ included in the sample. For both samples, there was no significant temperature difference (less than 1°C) between a ^{119}Sn 90°-observe experiment ($\times$ and $+$ symbols in Figure 4) and a TCUP-REDOR experiment with no decoupling (open triangles and circles in Figure 4).

The heating observed with decoupling is consistent with the expected freezing behavior of the two types of samples and further demonstrates the need for complete freezing to minimize heating of a high salt sample by the RF pulses. Although the DMSO-containing sample appears frozen at a sample temperature of -43°C , as judged by the probe low-power tuning behavior, DMSO forms very low freezing-point mixtures with water ($T_f = -63^\circ\text{C}$, for the pure 2-component system at its eutectic composition of $\approx 60\%$ DMSO), and so a portion of the DMSO-containing sample is likely to remain liquid.[22] This sample heats significantly ($\sim 17^\circ\text{C}$) at the lowest obtainable temperature (Figure 4, red triangles at $T_g = -60^\circ\text{C}$), suggesting that it is not fully frozen at the -43°C sample temperature. If the sample were a pure DMSO/water mixture, its 5% DMSO composition predicts that $\sim 92\%$ of it (all water) would freeze, leaving 8% at the eutectic composition of $\sim 60\%$ DMSO that would not freeze at $T_s = -43^\circ\text{C}$. Based on these known properties of DMSO/water mixtures, DMSO is not a good choice of cryoprotectant for a high salt sample that must be fully frozen to minimize heating by RF pulses.

The alternate sample components found to be effective cryoprotectants, 7.5% PEG 8000 and 4% trehalose, are predicted to be more compatible with the NMR experiment: the melting point for a water/PEG eutectic mixture is much higher, $\sim -15^\circ\text{C}$, with some dependence on the molecular weight of the polymer.[23] Consistent with this, the sample shows a constant small degree of heating ($4\text{--}6^\circ\text{C}$, Figure 4, filled blue circles) at low cooling gas temperatures of -60 and -50°C corresponding to sample temperatures of -44°C and -36°C , respectively. However there is greater heating ($\sim 13^\circ\text{C}$) at a cooling gas temperature of -40°C (sample temperature of -28°C), suggesting that the sample is not fully frozen. This reflects the fact that the -15°C melting temperatures for simple mixtures[23] cannot fully predict the properties of complex samples that contain additional salts, buffering agents etc, which demonstrates the importance of measuring sample heating under the conditions of interest. As observed for the DMSO sample, the PEG/trehalose-containing sample appears similarly frozen at all three temperatures (based on the probe low-power tuning behavior), further indicating that low-power tuning shifts are not diagnostic of whether a sample is fully frozen or will thaw under high power pulsing.

The combined effects that salt content and freezing point depression exert on the heating of the sample by RF pulses may not be widely appreciated. Samples of substantial ionic strength, but pulsed at temperatures where they remain solid, should experience relatively little heating. Samples that are liquid, yet contain little salt, should also incur little heating. However, samples that contain salt and that have at least some portion of the sample remaining liquid at the temperature of the NMR experiment can be expected to heat substantially. Molecules like DMSO and various polyols are used as cryoprotectants in other fields such as x-ray crystallography or tissue biology. Indeed, in our sample DMSO provided partial protection against freezing damage, when no RF pulses were applied. However, these compounds are usually added in the context of samples that are taken to liquid nitrogen temperatures, and which do not experience RF irradiation. Many of the chemicals used as cryoprotectants in those contexts either freeze, or form mixtures with water that freeze, at temperatures that may be difficult to maintain with standard solid-state NMR instrumentation. If the samples also contain salts, then the application of RF pulses can be expected to heat the sample, thereby melting a larger portion of the sample, so increasing its heat absorption, and so on, leading to significant sample heating. During any pause in the NMR experiment, the sample would refreeze until resumption of RF pulsing. Repeated freeze/thaw cycles or sample heating or both could irreversibly damage a typical protein sample.

Examples exist in the literature of solid-state NMR experiments performed on samples containing such traditional, low-melting point cryoprotectants. The Tycko lab has performed many experiments in ~50% glycerol/water mixtures (eutectic freezing point ~-40 °C, glass transition ~-70 °C), [24, 25]. However, in these cases, custom-built instrumentation was used to keep the sample unusually cold (~-150 °C). More typical conditions (cooling gas temperatures of ~-60 °C, corresponding to sample temperatures of ~-45 °C for our conditions) are quite close to the freezing point: small temperature increases with pulsing or over the course of a long experiment could partially thaw the sample, which would then lead to significant heating and thawing if the sample contains high salt. Although the protein might retain function if there are few freeze/thaw cycles (few pauses in the NMR pulsing) or if the sample conditions include adequate cryoprotectants (eg PEG), it is preferable to choose conditions that completely avoid sample thawing during the NMR experiment.

Careful attention to probe tuning properties can be used as a qualitative indicator of whether the NMR pulses thaw a significant fraction of the sample. Probe tuning properties differ for liquid and frozen aqueous samples. The observed tuning change (during low power tuning) upon freezing of the majority of the sample may suggest that the sample is fully frozen. Before performing any adjustment of the probe tuning under high power pulsing, the difference between the initial and steady state reflected power (relative to the total forward power) provides an indication of whether the pulses are thawing a large fraction of the sample. We used the peak-to-peak forward and reflected voltage to determine $\Delta V_{\text{REF}}/V_{\text{FWD}}$ as a measure of detuning, for samples containing 150 mM KCl and/or 5% DMSO. As shown in Figure 5, there is substantial detuning ($\Delta V_{\text{REF}}/V_{\text{FWD}}$ of 0.3–0.6) of the KCl-containing samples over temperature ranges where they are not fully frozen: $T_{\text{gas}} < -10$ °C for the KCl/DMSO sample (red squares) that never fully freezes, and a more limited range of $-20 < T_{\text{gas}} < -8$ °C for the KCl sample (black diamonds) that is expected to fully freeze at a sample temperature of -11 °C. Moderate detuning of these samples ($\Delta V_{\text{REF}}/V_{\text{FWD}}$ of 0.1–0.2) occurs at gas temperatures ~ -10 °C, due to sample heating with no phase change. These results are consistent with Figure 3: unfrozen KCl-containing samples experience RF-induced heating, which predicts significant probe detuning for the DMSO-containing sample at all observable temperatures until the sample is fully liquid, and for the KCl-only sample over the 11 °C temperature range in which it is partially frozen. Over the temperature range with significant detuning for the KCl-only sample ($\Delta V_{\text{REF}}/V_{\text{FWD}}$ of 0.3–0.6), post-pulsing

proton signals are significantly larger than pre-pulsing signals (data not shown), which confirms that pulses are thawing the water over this temperature range.

In contrast, the DMSO-only control sample (open circles) experiences negligible detuning at most temperatures: without KCl there is little heating-induced thawing of this sample in the high and low temperature ranges where the sample remains either 100% or about 8% unfrozen, respectively. As T_{gas} is raised above -15°C , the liquid water content of the DMSO-only sample gradually increases and there is sufficient rf-induced heating of this larger unfrozen volume to cause some additional thawing that detunes the probe. This detuning drops off when the sample is fully thawed because the small amount of heating in the absence of KCl does not significantly detune the probe.

Detuning measurements on intermediate concentrations of KCl and on a lipid vesicle-containing sample yielded similar data (Figure S1, supplementary material): probe detuning (Figure S1a) and significant post-pulsing increases in the proton signal (Figure S1b) occur over similar temperature ranges, consistent with the pulses thawing water in the sample and demonstrating that this method is viable for detecting thawing of a biological sample. Detuning and thawing begins at a lower temperature for the highest (150 mM) KCl concentrations, presumably because a larger fraction of the sample forms the eutectic and thaws at -11°C , causing more probe detuning and further heating and thawing. Note that the magnitude of the detuning is variable, with maxima ranging from $\Delta V_{\text{REF}}/V_{\text{FWD}}$ of 0.6 (Figure 5) to 0.14 (Figure S1a) for the same probe in different experiments on equivalent 150 mM KCl samples. Thus there is not always significant detuning during heating (eg 0 to 10°C range for Figure S1a) but there is always a clear detuning when the sample undergoes a pulsing-induced phase transition.

These results demonstrate that reflected power changes can be used to determine the temperature range over which the NMR experiment causes sample thawing. Measurements of tuning changes can be performed with the NMR experiment and sample composition of interest to provide an initial indicator of whether sample thawing is likely to be an issue and to empirically determine the temperature range over which the NMR experiment will cause thawing for this sample composition. This can inform the choice of NMR conditions such that small temperature increases over the course of a long experiment will not lead to sample thawing. Furthermore, careful attention to such tuning changes under initial pulsing during the actual experiment will also provide an indication of whether significant sample thawing is occurring.

Due to the complexity of biological sample conditions, actual freezing temperatures are difficult to predict. Empirical screening of conditions for a sacrificial sample, using the chemical shift thermometer to measure the degree of heating or measuring probe detuning to find the temperature range over which the sample is partially frozen, provides a practical approach to ensure that the buffer conditions are NMR-compatible before risking damage to a valuable sample.

2.3 More efficient sample cooling is a promising additional heat management strategy

Very rapid temperature measurements using the $\text{Sm}_2\text{Sn}_2\text{O}_7$ chemical shift thermometer make it possible to follow the time course of RF-induced heating. Because of the way the TCUP-REDOR experiment collects many ^{119}Sn spectra over the course of a single REDOR sequence, we were able to measure whether the temperature rises slowly over many pulses, or spikes quickly with each decoupling pulse and then cools again in the interim before the next one. A long recycle delay is used (10 s) to allow the sample to return to thermal equilibrium between each REDOR sequence. Instead of averaging signals from all of the ^{119}Sn spectra (as was done for Figures 3 and 4 to report an average sample temperature),

each of the 64 ^{119}Sn FIDs collected during the TCUP-REDOR sequence was averaged only with the corresponding FID in the subsequent REDOR sequence (ie, 10 s later), and *not* with the FID immediately following within a single REDOR sequence. In this way a series of ^{119}Sn FIDs are acquired, each one reporting on the temperature at a particular elapsed time into a single REDOR experiment. This experiment was performed on a sample containing protein-lipid complexes (the DMSO-containing sample of Figure 4) to yield the data plotted in Figure 6. These data show the time course of the sample temperature during each TCUP-REDOR sequence, starting with the 30 ms of ^1H decoupling highlighted in gray and continuing for about 170 ms after the decoupling stops.

The data show that the sample heats rapidly during the 30 ms of applied ^1H decoupling (gray), but only by $\sim 1.5^\circ\text{C}$. The sample then cools very slowly during the recycle delay, over time scales not measured by our pulse program. Cooling during the 10 s recycle delay brings the sample back to the baseline temperature, as demonstrated by the fact that the starting temperature in Figure 6 is equivalent to that obtained with MAS alone (no decoupling) in Figure 1 (equation predicts $T_{\text{sample}} = -35.7^\circ\text{C}$). This implies that the large amounts of heating observed in Figure 4 are *not* due to rapid heating during a single REDOR pulse sequence; rather, they are the cumulative results of many such sequences, repeated too quickly (delay of 1 s used for Figure 4 experiments) for the sample to return to thermal equilibrium between them. Thus the heating of the sample is attributable at least as much to ineffective removal of heat by the cooling system, as it is to excessive absorption of heat by the sample: if the cooling system were more effective, the sample might only ever experience the 1.5°C heating depicted in Figure 6. To date, most schemes to mitigate RF induced heating have focused on limiting the absorption of heat, either by limiting salt content in the sample or by constructing coils that produce smaller electric fields for a given magnetic strength.[9, 16, 26] However, the data presented here imply that improving the performance of the cooling system may be at least as effective.

We used a static NMR sample to test whether high cooling gas flow rates more effectively dissipate the RF pulse heating of the sample. Sample temperatures were monitored using 20 mM TmDOTP as an internal chemical shift thermometer[27] in an unfrozen high ionic strength sample (150 mM NaCl, 10% DMSO). Monitoring the ^{31}P signal in TmDOTP is more convenient than retuning the probe to ^{119}Sn , and we reasoned that a soluble compound is adequate for this application since its effect on freezing properties is irrelevant and its perturbation to the ionic strength is small. Figure 7 shows that increasing the flow rate of the cooling gas does indeed reduce the cumulative heating of the sample by multiple pulses: the heating due to decoupling is much smaller with higher cooling gas flow rate (180 scfm, center dotted spectrum) as demonstrated by the smaller chemical shift from the no-decoupling spectrum at 0 ppm. With the $+2.18\text{ ppm}/^\circ\text{C}$ value previously determined for ^{31}P in TmDOTP, [27] we used the dominant downfield peak to estimate the maximum heating and the smaller upfield component to estimate the temperature gradient for each spectrum. Multiple pulses with decoupling heated the sample by 23°C with 100 scfh cooling gas but by only 12°C with 180 scfh cooling gas. The higher gas flow also reduced the temperature gradient across the sample from about 17°C (100 scfh) to 11°C (180 scfh).

These data indicate that increased sample cooling efficiency is an important strategy to pursue for efficient NMR of functional samples, so that the repetition rate of the experiment is limited only by power handling of the probe and NMR relaxation times rather than by sample cooling considerations. Increasing cooling gas flow rates is not an ideal solution, since the gas cannot be cooled as effectively at higher flow rates (180 scfh gas is cooled to only -11°C with our FTS cooling system). Alternatively, increases in sample cooling efficiency may be achieved by rotor designs that improve heat-exchange characteristics, for

instance by increasing the surface area, thinning the walls, or using materials with higher heat conductivity.

3. Conclusion

For NMR studies of frozen samples, it is critical to identify conditions under which proteins remain functional after both the freezing and the pulsing required for the NMR experiment. Using a small-scale biochemical assay of slow-freezing induced damage we identified conditions containing PEG 8000 and trehalose that preserve activity of a chemoreceptor signaling complex.[21] The $\text{Sm}_2\text{Sn}_2\text{O}_7$ -monitored heating experiments performed on a low-cost sacrificial sample containing the buffer salts and cryoprotectant conditions that preserve function (Figure 4) demonstrate that the NMR conditions (sample temperature, pulse power and duty cycle, MAS speed, etc) do not thaw or significantly heat such a sample. The success of this approach is demonstrated by activity measurements: the kinase activity of the complex is essentially the same before and after the REDOR NMR experiments.[21] Thus internal temperature standards in representative samples can be used to identify robust conditions for solid-state NMR of functional protein complexes.

To minimize perturbation of the freezing and heat absorption properties of the sample, an insoluble internal temperature standard such as $\text{Sm}_2\text{Sn}_2\text{O}_7$ provides the best approach to accurately monitor the temperature under the conditions of interest for NMR studies of frozen samples. In light of the substantial thermal gradients that have been measured for some samples (eg 12°C for a 165 mM ionic strength sample in a solenoid coil),[9] an internal standard provides the most accurate information to identify experimental conditions that prevent sample damage. Although $\text{Sm}_2\text{Sn}_2\text{O}_7$ is a better reporter of axial temperature gradients than external KBr located at one end of the sample, it may not be an accurate reporter of radial temperature gradients: it is anticipated that MAS forces will sometimes lead to radial separation from the sample, with the high density $\text{Sm}_2\text{Sn}_2\text{O}_7$ at the perimeter of the rotor. Furthermore, use of $\text{Sm}_2\text{Sn}_2\text{O}_7$ requires retuning the probe to the resonant frequency of ^{119}Sn , which may not be possible in all cases and precludes monitoring the temperature during the actual NMR experiment. The method reported by Thurber et al using a separate plug of KBr at the top of the sample[6] represents a convenient compromise: the temperature measurement may not be as accurate as with $\text{Sm}_2\text{Sn}_2\text{O}_7$ if there are thermal gradients or limitations in heat transfer, but only minor retuning is required since the resonant frequency of ^{79}Br is close to that of ^{13}C .

An even simpler approach provides critical information for experiments on frozen samples: a qualitative assessment of sample thawing due to RF-induced heating can be made by measuring changes in reflected voltage on a sacrificial sample or even a suitable mixture of buffer, salt, and cryoprotectant. Samples that undergo melting exhibit pronounced detuning, while samples that experience heating exhibit relatively modest increases in reflected voltage. This allows for rapidly determining sample and temperature conditions that are compatible with the NMR experiment, as well as monitoring for problems during the actual experiment.

Such temperature and detuning measurements on low-cost samples are essential to identify NMR and sample conditions that allow the most efficient collection of NMR data (eg the shortest recycle delay, with power levels and MAS speeds needed for resolution) while preserving the sample functionality. Such optimization is especially important when functional conditions require significant salt concentrations. We have also shown that single scans cause limited sample heating, so improving the cooling efficiency is another promising approach that could enable more efficient data acquisition with short recycle

delays. These approaches should facilitate expansion of solid-state NMR studies to more proteins and protein complexes in their functional states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Luo W, Cady SD, Hong M. Immobilization of the influenza A M2 transmembrane peptide in virus envelope-mimetic lipid membranes: a solid-state NMR investigation. *Biochemistry*. 2009; 48:6361–6368. [PubMed: 19489611]
2. Lorieau JL, Day LA, McDermott AE. Conformational dynamics of an intact virus: order parameters for the coat protein of Pf1 bacteriophage. *Proc Natl Acad Sci U S A*. 2008; 105:10366–10371. [PubMed: 18653759]
3. Tycko R. Solid-state NMR studies of amyloid fibril structure. *Annu Rev Phys Chem*. 2011; 62:279–299. [PubMed: 21219138]
4. Van Melckebeke H, Wasmer C, Lange A, Ab E, Loquet A, Bockmann A, Meier BH. Atomic-resolution three-dimensional structure of HET-s(218–289) amyloid fibrils by solid-state NMR spectroscopy. *J Am Chem Soc*. 2010; 132:13765–13775. [PubMed: 20828131]
5. Dvinskikh SV, Castro V, Sandstrom D. Heating caused by radiofrequency irradiation and sample rotation in ^{13}C magic angle spinning NMR studies of lipid membranes. *Magn Reson Chem*. 2004; 42:875–881. [PubMed: 15366061]
6. Thurber KR, Tycko R. Measurement of sample temperatures under magic-angle spinning from the chemical shift and spin-lattice relaxation rate of ^{79}Br in KBr powder. *J Magn Reson*. 2009; 196:84–87. [PubMed: 18930418]
7. Brus J. Heating of samples induced by fast magic-angle spinning. *Solid State Nucl Magn Reson*. 2000; 16:151–160. [PubMed: 10868566]
8. Dillmann B, Elbayed K, Zeiger H, Weingertner MC, Piotto M, Engelke F. A novel low-E field coil to minimize heating of biological samples in solid-state multinuclear NMR experiments. *J Magn Reson*. 2007; 187:10–18. [PubMed: 17448715]
9. Stringer JA, Bronnimann CE, Mullen CG, Zhou DH, Stellfox SA, Li Y, Williams EH, Rienstra CM. Reduction of RF-induced sample heating with a scroll coil resonator structure for solid-state NMR probes. *J Magn Reson*. 2005; 173:40–48. [PubMed: 15705511]
10. Langer B, Schnell L, Spiess HW, Grimmer AR. Temperature calibration under ultrafast MAS conditions. *J Magn Reson*. 1999; 138:182–186. [PubMed: 10329244]
11. Doty FD, Kulkarni J, Turner C, Entzminger G, Bielecki A. Using a cross-coil to reduce RF heating by an order of magnitude in triple-resonance multinuclear MAS at high fields. *J Magn Reson*. 2006; 182:239–253. [PubMed: 16860580]
12. Gor'kov PL, Chekmenev EY, Li CG, Cotten M, Buffy JJ, Traaseth NJ, Veglia G, Brey WW. Using low-E resonators to reduce RF heating in biological samples for static solid-state NMR up to 900 MHz. *J Magn Reson*. 2007; 185:77–93. [PubMed: 17174130]
13. Gor'kov PL, Witter R, Chekmenev EY, Nozairov F, Fu R, Brey WW. Low-E probe for F-19-H-1 NMR of dilute biological solids. *J Magn Reson*. 2007; 189:182–189. [PubMed: 17920316]
14. Grant CV, Sit SL, De Angelis AA, Khuong KS, Wu CH, Plesniak LA, Opella SJ. An efficient H-1/P-31 double-resonance solid-state NMR probe that utilizes a scroll coil. *J Magn Reson*. 2007; 188:279–284. [PubMed: 17719813]

15. Krahn A, Priller U, Emsley L, Engelke F. Resonator with reduced sample heating and increased homogeneity for solid-state NMR. *J Magn Reson.* 2008; 191:78–92. [PubMed: 18187352]
16. McNeill SA, Gor'kov PL, Shetty K, Brey WW, Long JR. A low-E magic angle spinning probe for biological solid state NMR at 750 MHz. *J Magn Reson.* 2009; 197:135–144. [PubMed: 19138870]
17. Mildner T, Ernst H, Freude D. 207Pb NMR detection of spinning-induced temperature gradients in MAS rotors. *Solid State Nucl Magn Reson.* 1995; 5:269–271. [PubMed: 9053118]
18. Campbell GC, Crosby RC, Haw JF. ¹³C Chemical shifts which obey the Curie Law in CP/MAS NMR spectra. The first CP/MAS NMR chemical-shift thermometer. *J Magn Reson.* 1986; 69:191–195.
19. Zuo CS, Bowers JL, Metz KR, Nosaka T, Sherry AD, Clouse ME. TmDOTP5-: a substance for NMR temperature measurements in vivo. *Magn Reson Med.* 1996; 36:955–959. [PubMed: 8946362]
20. Grey CP, Cheetham AK, Dobson CM. Temperature-Dependent Solid-State Sn-119 Mas Nmr of Nd₂Sn₂O₇, Sm₂Sn₂O₇, and Y_{1.8}Sm_{0.2}Sn₂O₇ - 3 Sensitive Chemical-Shift Thermometers. *Journal of Magnetic Resonance Series A.* 1993; 101:299–306.
21. Fowler DJ, Weis RM, Thompson LK. Kinase-active signaling complexes of bacterial chemoreceptors do not contain proposed receptor-receptor contacts observed in crystal structures. *Biochemistry.* 2010; 49:1425–1434. [PubMed: 20088541]
22. Rasmussen DH, MacKenzie AP. Phase diagram for the system water-dimethylsulphoxide. *Nature.* 1968; 220:1315–1317. [PubMed: 5701346]
23. Hager SL, Macrury TB. Investigation of Phase-Behavior and Water Binding in Poly(Alkylene Oxide) Solutions. *Journal of Applied Polymer Science.* 1980; 25:1559–1571.
24. Sharpe S, Kessler N, Anglistter JA, Yau WM, Tycko R. Solid-state NMR yields structural constraints on the V3 loop from HIV-1 Gp120 bound to the 447-52D antibody Fv fragment. *J Am Chem Soc.* 2004; 126:4979–4990. [PubMed: 15080704]
25. Thurber KR, Tycko R. Biomolecular solid state NMR with magic-angle spinning at 25K. *J Magn Reson.* 2008; 195:179–186. [PubMed: 18922715]
26. Wu CH, Grant CV, Cook GA, Park SH, Opella SJ. A strip-shield improves the efficiency of a solenoid coil in probes for high-field solid-state NMR of lossy biological samples. *J Magn Reson.* 2009; 200:74–80. [PubMed: 19559634]
27. Zuo CS, Metz KR, Sun Y, Sherry AD. NMR temperature measurements using a paramagnetic lanthanide complex. *J Magn Reson.* 1998; 133:53–60. [PubMed: 9654468]

Highlights

Heating tests on a sacrificial sample enable efficient NMR of functional proteins.

$\text{Sm}_2\text{Sn}_2\text{O}_7$ chemical shift thermometer doesn't alter freezing point or ionic strength.

Complete freezing of high salt samples minimizes heating.

Simple tests for thawing during pulsing are described.

Increased cooling efficiency is an untapped method for effective heat management.

Figure 1.

Baseline sample temperatures during 5 kHz MAS established with $\text{Sm}_2\text{Sn}_2\text{O}_7$. Black triangle data were obtained in a Varian 4 mm HFX T3 probe. The sample temperature is calculated using equation (1) from the ^{119}Sn isotropic chemical shift measured at each cooling gas temperature (after equilibration for ~10 min). A linear fit (dashed black line: $T_s (^{\circ}\text{C}) = 0.78 T_g + 7.22$) to these data has slope less than 1 (gray line) and demonstrates there is very minor heating due to the 5 kHz MAS. Very similar baseline temperature data were obtained in a Varian 4 mm HXY APEX probe on the $\text{Sm}_2\text{Sn}_2\text{O}_7$ sample (inverted gray triangles) and on a mixture of $\text{Sm}_2\text{Sn}_2\text{O}_7$ and extruded DMPC vesicles in 150 mM KCl (circles). The ^{119}Sn chemical shift was referenced to SnO_2 at -604.3 ppm. All NMR experiments were performed on a Varian Infinity Plus spectrometer operating at a ^1H frequency of 300.1 MHz and ^{119}Sn frequency of 111.9 MHz.

Figure 2. Modified REDOR sequence for Temperature Calibration Under Pulse load: TCUP-REDOR. ^{119}Sn pulses replace the observe channel pulses of the REDOR experiment, and multiple acquisitions (X) are possible due to the short T_1 .

Figure 3.

Effect of freezing on RF-induced heating of a high ionic strength sample. ^{119}Sn spectra of ~ 17 mg $\text{Sm}_2\text{Sn}_2\text{O}_7$ in 150 mM KCl at initial sample temperatures of (A) 6 °C or (B) -38 °C. The liquid sample heats, which causes a chemical shift change, while the solid sample does not. TCUP-REDOR sequence of Figure 2 was used (red spectra) with $X=64$ and $n=56$. Each ^{119}Sn acquisition was 3 ms long, with a dwell time of 10 μs . Partial T_1 relaxation occurred during the 100 μs inserted between the end of one acquisition and the start of another. The blocks in parenthesis on the ^{19}F channel were synchronized to the rotor period, which was 200 μs . REDOR spectra included 100 kHz proton decoupling for a total of 30.4 ms, with a 1 s recycle delay. The proton decoupling is the dominant source of sample heating, since 56 rotor cycles of ^{19}F pulses sum to less than 1% of the decoupling time. Therefore, to test heating effects at the maximum proton decoupling power, the ^{19}F power was set to zero for the experiments shown in Figures 3, 4, and 6. Spectra are the sum of 256 scans each of REDOR S and S_0 (i.e. $256 \times 2 \times 64 = 32,768$ individual ^{119}Sn free induction decays (FIDs)), collected after 16 dummy scans for thermal equilibration in a Varian 4 mm HFX T3 probe.

Figure 4.

Heating of sample in different buffers, under different RF loads. Samples of lipid/protein complex containing $\text{Sm}_2\text{Sn}_2\text{O}_7$ and two different cryoprotectants are subjected to TCUP versions of specified pulse sequence: PEG (blue circles and $\times$ symbols) = 7.5% (w/v) PEG 8000 and 4% (w/v) trehalose; DMSO (red triangles and cross symbols) = 5% (v/v) DMSO. Both samples contain 75 mM potassium phosphate buffer, pH 8.0, 50 mM KCl. Significant sample heating occurs when 30 ms of 80 kHz proton decoupling is applied to samples that are not fully frozen: DMSO sample at $T_s = -43^\circ\text{C}$ without decoupling (open red triangle or cross) heats to -26°C with decoupling (filled red triangle); PEG sample at $T_s = -28^\circ\text{C}$ without decoupling (open blue circle or $\times$ symbol) heats to -14°C with decoupling (filled blue circle). Each point is determined from a spectrum derived from 512 scans each of REDOR S and S_0 , each with 32 individual ^{119}Sn FIDs in a Varian 4 mm HFX T3 probe.

Figure 5.

Probe detuning measurements identify temperature range over which RF pulsing causes sample phase changes. The change from initial to steady state reflected voltage (ΔV_{REF}) on the ^1H channel is used to estimate probe detuning, which is expressed as a ratio of $\Delta V_{\text{REF}} / V_{\text{FWD}}$. This is plotted as a function of T_{gas} for 150 mM KCl/ H_2O (black diamonds), 150 mM KCl/ H_2O /5 % DMSO (red squares), and H_2O /5 % DMSO (open circles). A standard $^1\text{H}/^{13}\text{C}$ cross polarization experiment was used for 400 scans, 29 ms acquisition time, 83 kHz ^1H decoupling, and 1 second recycle time, on a Varian 4 mm HXY Apex probe. The sample was equilibrated for 10 min at each value of T_{gas} prior to pulsing.

Figure 6.

Time course of RF heating of unfrozen high salt sample during NMR. Sample temperature determined from ^{119}Sn chemical shift in a sample containing protein-lipid complex in 5% DMSO, as in Figure 4. Temperatures for each timepoint were determined from spectra obtained from 6144 scans each REDOR S and S_0 . A 10 s recycle delay was used. In each scan, a total of 30 ms of 80 kHz ^1H decoupling was applied in a Varian 4 mm HFX T3 probe. The temperature appears to jump incrementally due to limited digital resolution ($0.5^\circ\text{C} \approx 90\text{ Hz}$). The temperature of the cooling gas used was -55°C ; a resting temperature of -36°C is consistent with the cooling curve shown in Figure 1, indicating the sample returns to thermal equilibrium between each scan.

Figure 7.

^{31}P spectra illustrate heating of a static, TmDOTP-doped, high ionic strength sample. Cooling gas at 0 °C was supplied at 100 (left black spectrum) or 180 (middle dotted spectrum) standard cubic feet per hour to a sample of 150 mM NaCl, 10% DMSO. ^1H decoupling was 57 kHz and was applied for 20.48 ms per scan with a 1 s recycle delay in a Varian 4 mm HXY Apex probe; steady state heating was measured since at least 64 scans were collected. Chemical shifts are referenced with 0 ppm being the shift of the ^{31}P resonance in an experiment performed without ^1H decoupling (right gray spectrum).