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(Instructions) High-pressure NMR experiments for detecting protein low-lying conformational states

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TITLE:

High-Pressure NMR Experiments for Detecting Protein Low-Lying Conformational States

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KEYWORDS:

high-pressure NMR, protein folding, protein stability, high-energy conformational states

SUMMARY:

We provide a detailed description of the steps required to assemble a high-pressure cell, set up and record high-pressure NMR experiments, and finally analyze both peak intensity and chemical shift changes under pressure. These experiments can provide valuable insights into the folding pathways and structural stability of proteins.

ABSTRACT:

High-pressure is a well-known perturbation method that can be used to destabilize globular proteins and dissociate protein complexes in a reversible manner. Hydrostatic pressure drives thermodynamical equilibria toward the state(s) with the lower molar volume. Increasing pressure offers, therefore, the opportunities to finely tune the stability of globular proteins and the oligomerization equilibria of protein complexes. High-pressure NMR experiments allow a detailed characterization of the factors governing the stability of globular proteins, their folding mechanisms, and oligomerization mechanisms by combining the fine stability tuning ability of pressure perturbation and the site resolution offered by solution NMR spectroscopy. Here we present a protocol to probe the local folding stability of a protein via a set of 2D 1H-15N experiments recorded from 1 bar to 2.5 kbar. The steps required for the acquisition and analysis of such experiments are illustrated with data acquired on the RRM2 domain of hnRNPA1.

INTRODUCTION:

It has long been recognized that higher-energy, sparsely populated conformational states of proteins and protein complexes play a key role in many biological pathways[1–3]. Thanks to experiments based on Carr-Purcell-Meiboom-Gill (CPMG)[4], Chemical Exchange Saturation

Transfer (CEST)[5] and dark-state exchange saturation transfer (DEST)[6] pulse sequences (among others), solution NMR spectroscopy has emerged as a method of choice for characterizing transient conformational states[7]. Along with these experiments, perturbations such as temperature, pH, or chemical denaturants can be introduced to increase the relative population of higher energy conformational substates. Similarly, protein equilibria can also be perturbed by applying high hydrostatic pressure. Depending on the magnitude of the volume change associated with the corresponding conformational changes, an increase of pressure by a few hundred to a few thousand bars can significantly stabilize a higher energy state or cause a protein to completely unfold[8–10]. Protein NMR spectra typically display two types of changes with hydrostatic pressure: (i) chemical shift changes and (ii) peak intensity changes. Chemical shift changes reflect changes at the protein surface-water interface and/or local compression of the protein structure on a fast time scale (relative to NMR time scale)[11]. Crosspeaks exhibiting large non-chemical shifts pressure dependence can indicate the presence of higher energy conformational states[12,13]. On the other hand, peak intensity changes point to major conformational transitions on a slow time scale, such as changes in folded/unfolded state populations. The presence of folding intermediates or higher energy states can be detected from large variations in the magnitude of the volume change upon unfolding measured for different residues of a given protein[14–17]. Based on experience, even small proteins that are typically classified as two-state folders exhibit non-uniform responses to pressure, which provides useful information about their local folding stability. Described here is a protocol for the acquisition and analysis of amide peak intensity and ^{1}H chemical shifts pressure dependence, using as a model protein the isolated RNA recognition motif 2 (RRM2) of the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1).

PROTOCOL:

NOTE: The protocol described here requires (i) a high-pressure pump and cell with a 2.5 kbar rated aluminum-toughened zirconia tube[18], (ii) the software SPARKY[19] for analysis of the NMR spectra, and (iii) a curve fitting software.

1. Sample preparation, assembly of the high-pressure cell, and setting up the experiments.

1.1. Choice of buffer: Use equal mixture of anionic and cationic buffers, such as phosphate and Tris[20,21].

NOTE: The pKa of anionic buffers such as phosphate and MES is associated with a substantial reaction volume (i.e., the difference in the partial molal volumes of the acid and the ionized products). The pH of such buffers can therefore be significantly affected by a change of pressure (~0.25–0.5 pH unit/kbar).

1.2. Ensure that the required sample volume is similar to that of a standard 3 mm diameter NMR tube (~300 µL).

1.3. Introduce the ^{15}N-labeled sample with a glass pipette into the zirconia tube. Make sure the sample seats at the bottom of the tube. Complete with 200 µL of mineral oil to prevent the sample from mixing with the transmission liquid (e.g., water). Fill the rest of the tube with transmission liquid.

1.4. Put a single-use O-ring on top of the zirconia tube and slide the tube into the base (**Figure 1A**,**B**). Then, connect the tube to the high-pressure tether line and tighten the base to the cell first by hand. Then, apply 14.7 Nm of torque to prevent leaks at lower pressure (**Figure 1C**,**D**).

1.5. To check the integrity of the pressure cell assembly, pressurize the tube up to 300 bar outside the spectrometer using cell support and containment vessel. Wait for 15 min, reset the pressure to 1 bar and check for leaks with a clean lint-free wipe.

1.6. Insert the unpressurized tube into the spectrometer by carefully guiding the tether line. Slide the tube in the spectrometer until reaching the sample sitting position (**Figure 1E**).

1.7. Lock, shim, match, and tune the ^{1}H and ^{15}N channels as usual.

NOTE: Shims for high-pressure rated zirconia tubes are very different from standard NMR tubes. It is recommended to save the optimized shims for future use.

1.8. Set up a ^{1}H-^{15}N-HSQC or TROSY-HSQC and record a reference experiment at atmospheric conditions (1bar).

2. Recording high-pressure NMR experiments

2.1. Gradually increase the pressure from 1 bar to 2.5 kbar with 500 bar increments to test the overall stability of the protein. Set the speed of the pressure pump by default at ~18 bar/s. If the precise folding/unfolding rates are not known, let the sample equilibrate 15–20 min after each 500-bar increment. Record a spectrum at 2.5 kbar.

2.2. Gradually decrease the pressure back to 1 bar with 500 bar steps to test the reversibility of the pressure perturbation. Record another spectrum at atmospheric conditions and compare the chemical shifts and peak intensities with that of the reference spectrum previously recorded in the same conditions.

NOTE: If the native crosspeaks are more intense after the pressure run, it may indicate that small aggregates present in solution at atmospheric pressure may have dissociated and properly refolded. On the other hand, a loss in the intensity or significant chemical shift changes suggest that the protein may experience a non-reversible misfolding in high-pressure conditions.

2.3. Record a series of 2D experiments from 1 bar to 2.5 kbar every 500 bar. It is recommended to record additional experiments near the inflection point of the folding/unfolding transition to improve the precision of the fit.

3. Analyzing peak intensity changes

3.1. Process all the spectra and transfer the backbone assignment from the reference spectrum at 1 bar to the spectrum recorded at 500 bar, and then transfer the assignment from 500 bar to 1 kbar and so forth.

NOTE: Because pressure induces a non-uniform shift of ^{1}H and ^{15}N chemical shifts, do not simply copy the backbone assignment from one spectrum to the next. Adjust it manually.

3.2. In Sparky menu, click on **Peak** > **Peak List (lt)**. In the **Peak List** window, click on **Options** and select the option to display both **Frequencies (Ppm)** and **Data Height**. Save the list obtained for each spectrum.

3.3. In a curve fitting software, copy the crosspeak identity and peak intensity values to have the pressure values (in bar) as the X-axis variable and the intensity as the Y-axis variable.

3.4. If a complete or near complete (>80%) unfolding is observed, fit the individual peak intensity profiles to extract the free energy and volume change upon unfolding, respectively, using a simple two-state model:

$$I = \frac{I_F}{1+e^{-[\Delta G_U^0 + \Delta V_U (p-p_0)]/RT}} \qquad \text{Eq. 1}$$

Where, "I" is the observed intensity of a crosspeak at a given pressure p and I_F is the intensity of the same crosspeak in a fully folded state. R is the gas constant, T is the absolute temperature, ΔG_U^0 the standard Gibbs free energy difference between the unfolded and folded states at atmospheric pressure p_0 (1 bar), and ΔV_u the volume change upon unfolding. With the pressure p in bar and temperature T in kelvin, R = 1.987 cal/K, ΔG_U^0 is in cal/mol, and ΔV_U is in cal/mol/bar. Multiply the ΔV_U values obtained from the fit by 41.84 to convert into mL/mol. ΔV_U values are typically in the range of -50 to -150 mL/mol for globular proteins[22]. Here, all the parameters are expressed with regard to the unfolding reaction but can easily be converted into folding reaction parameters ($\Delta G_U^0 = -\Delta G_F^0$ and $\Delta V_U = -\Delta V_F$).

4. Analyzing chemical shift changes

4.1. Arrange the columns in the software in order to have pressure points as variable and the ^{1}H chemical shifts extracted from Sparky lists as the Y-axis.

4.2. Fit the pressure dependence of the ^{1}H chemical shifts to a simple quadratic equation:

$$\delta(p) = \delta_0(p_0) + B_1(p-p_0) + B_2\,(p-p_0)^2 \qquad \text{Eq. 2}$$

Where, $\delta(p)$ is the measured ^{1}H chemical shift of a crosspeak at a given pressure p and $\delta_0(p_0)$ the ^{1}H chemical shifts of the same crosspeak in the reference spectrum recorded at 1 bar. B_1 and B_2 represent the first and second-order parameters expressed in ppm/bar and ppm/bar^2, respectively.

REPRESENTATIVE RESULTS:

The protocol described here was used to probe the pressure dependence of RRM2, the second RNA recognition motif hnRNPA1 (residues 95–106), which is almost completely unfolded within the 2.5 kbar range (>90%). ^{1}H-^{15}N spectra were collected at 1 bar, 500 bar, 750 bar, 1 kbar, 1.5 kbar, 2 kbar, and 2.5 kbar bar (**Figure 2**). Since none of the native crosspeaks were visible above the noise level at 2.5 kbar bar, all corresponding residues were attributed an intensity value of 0 at this pressure (**Figure 3A**). A total of 45 individual pressure intensity profiles were fitted according to Equation 1 (Eq. 1) to obtain the corresponding changes in standard Gibbs free-energy (ΔG_U^0) and volume (ΔV_U) associated with the unfolding reaction (**Figure 3A**). The calculated ΔV_U values ranged from -41 to -88 mL/mol and ΔG_U^0 from 1.8–3.3 kcal/mol. When mapped on the domain structure (pdb 1U1R), it appears that residues with the largest magnitude of volume change are found within the domain structural core (in red in **Figure 3B**), while those with the smallest magnitude of volume change are mainly located in the loop connecting the β-strands 2 and 3, and the loop connecting β-strand 4 to the C-terminal helix (in green, **Figure 3B**). Pressure dependence of the native backbone ^{1}H chemical shifts was also analyzed to probe the degree of compressibility and conformational heterogeneity of the folded state ensemble. Individual profiles of ^{1}H chemical shifts as a function of pressure were fitted using Eq. 2 in order to extract site-specific linear (B_1) and non-linear (B_2) coefficients (**Figure 3C**). The residues with the largest non-linear coefficients were mostly found in the structural core of the domain (in yellow, **Figure 3D**), while residues with the smallest non-linear coefficients were mostly located within loops connecting the different structural motifs (in blue, **Figure 3D**).

FIGURE AND TABLE LEGENDS:

Figure 1: High-pressure cell assembly and installation in the spectrometer. (A–D) The full high-pressure cell assembly requires a zirconia tube, a single-use O-ring, the cell base (**A,B**). The tube is connected to the tether line by tightening the base to the cell (**C,D**). 14.7 Nm of torque is required to prevent leaks at lower pressure. (**E**) The zirconia tube connected to the Xtreme-60 Syringe pump via the tether line is introduced into the spectrometer until reaching the normal sample sitting position.

Figure 2: Unfolding of hnRNPA1 RRM2 domain under pressure. ^{1}H-^{15}N HSQC spectra collected at (**A**) 1 bar, (**B**) 1,000 bar, (**C**) 1,500 bar, (**D**) and 2,500 bar show a complete unfolding of the RRM2 domain under pressure.

Figure 3: Analysis of RRM2 peak intensity and ^{1}H chemical shifts pressure dependence. (**A**) Representative peak intensity profiles recorded for RRM2 between 1 and 2,000 bar. Solid lines represent the fit obtained using Eq. 1 to calculate the corresponding standard free-energy and volume changes associated with the unfolding reaction. (**B**) The top 25% of residues with the largest measured magnitude of ΔV_U are highlighted with red spheres on RRM2 reference structure (pdb 1U1R). Residues with the smallest magnitude of ΔV_U are shown in green. (**C**) Representative changes of ^{1}H chemical shifts measured for RRM2 between 1 bar and 2,000 bar. Solid lines represent the fit to Eq. 2 to calculate the linear (B_1) and non-linear (B_2) pressure coefficients. (**D**) The top 25% of residues with the largest B_2 coefficients are shown in yellow, while those with the smallest B_2 coefficients are shown in blue.

DISCUSSION:

The study details a protocol implemented to probe protein structural and thermodynamics responses to pressure perturbation. The high-pressure experiments recorded here on RRM2 demonstrate that large variations in ΔV_U values, indicative of non-fully cooperative unfolding, can be found in a relatively small single domain protein. A similar picture emerges from the analysis of ^{1}H chemical shift changes under pressure. It should be noted Kalbitzer and coworkers have demonstrated that a more in-depth analysis of chemical shift changes can be performed linking the ratio between the non-linear and linear coefficients (B_2/B_1) with the ratio of the change in compressibility and volume ($\Delta\beta/\Delta V$)[23]. These results highlight the ability of high hydrostatic pressure to destabilize individual structural motifs and subdomains based on the local thermodynamic stability and volume change associated with unfolding. Recording a simple set of pressure titration experiments can therefore provide valuable information regarding the folding mechanisms and subdomain architecture of a protein.

The whole pressure system (pump and controller) is relatively compact and can be installed on a standard utility cart for easy access to the spectrometer (**Figure 1**). All pulse sequences can be used under pressure without modification, including triple resonance experiments. The only limitation is that pressure-rated zirconia tubes reduce the sensitivity of NMR experiments by almost 50% compared to a standard 3 mm diameter NMR tube. This may present a challenge for protein samples that can't be concentrated to a high-enough level. Compared to other perturbation methods such as temperature or chemical denaturants, hydrostatic pressure presents the advantage of being, with very few exceptions, fully reversible. Because pressure favors the dissociation of protein complexes, there is, for example, very little risk of sample aggregation, a problem often encountered with high temperatures.

While hydrostatic pressure is a powerful tool for studying protein stability, it is important to keep in mind that there is no perfect method of perturbation. Each of them highlights the specificity of a protein free-energy landscape in a different way. For example, if two conformational states have the same molar volume, an increase in hydrostatic pressure will not significantly change their relative populations. Similarly, if two conformational states have a similar exposed surface area, adding chemical denaturants would not necessarily increase the relative population of one state compared to the other one. Ideally, different perturbation methods should be combined to obtain a complete description of a protein free-energy landscape[15,24]. Finally, it should be noted

that although the manuscript is focused on pressure dependence of intensity and chemical shifts from series of 2D ^{1}H-^{15}N experiments measured at equilibrium, a wide range of NMR experiments can be coupled to pressure perturbation to probe different aspects of the stability, the folding mechanism, and the conformational dynamics of protein and protein complexes[20,25].

ACKNOWLEDGMENTS:

This work was supported by funds from the Roy J. Carver Charitable Trust to Julien Roche. We thank J. D. Levengood and B. S. Tolbert for kindly providing the RRM2 sample.

DISCLOSURES:

All the authors have read and approved the manuscript. They declare no conflicts of interest.

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Figure1

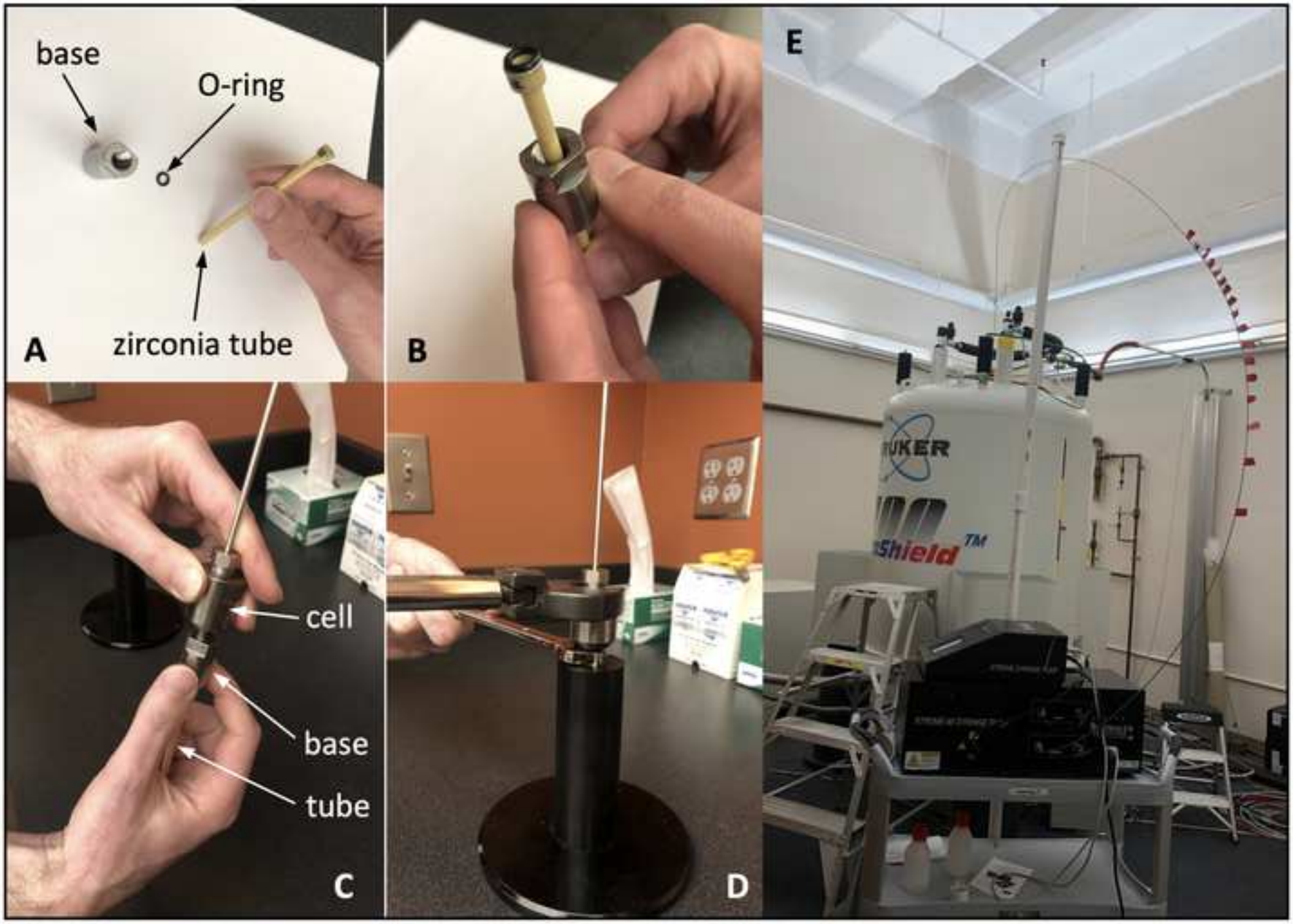

Figure2

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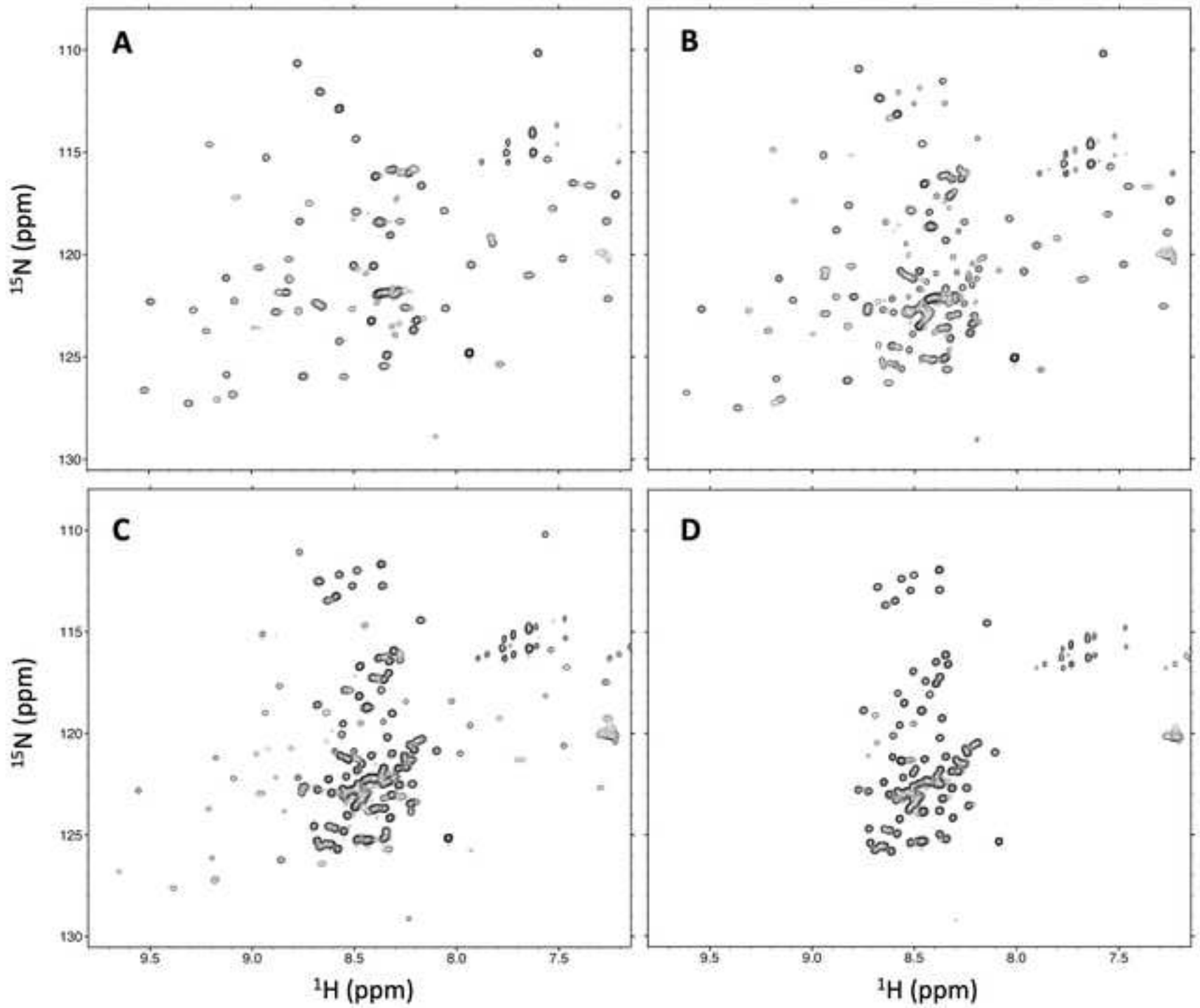

Figure3

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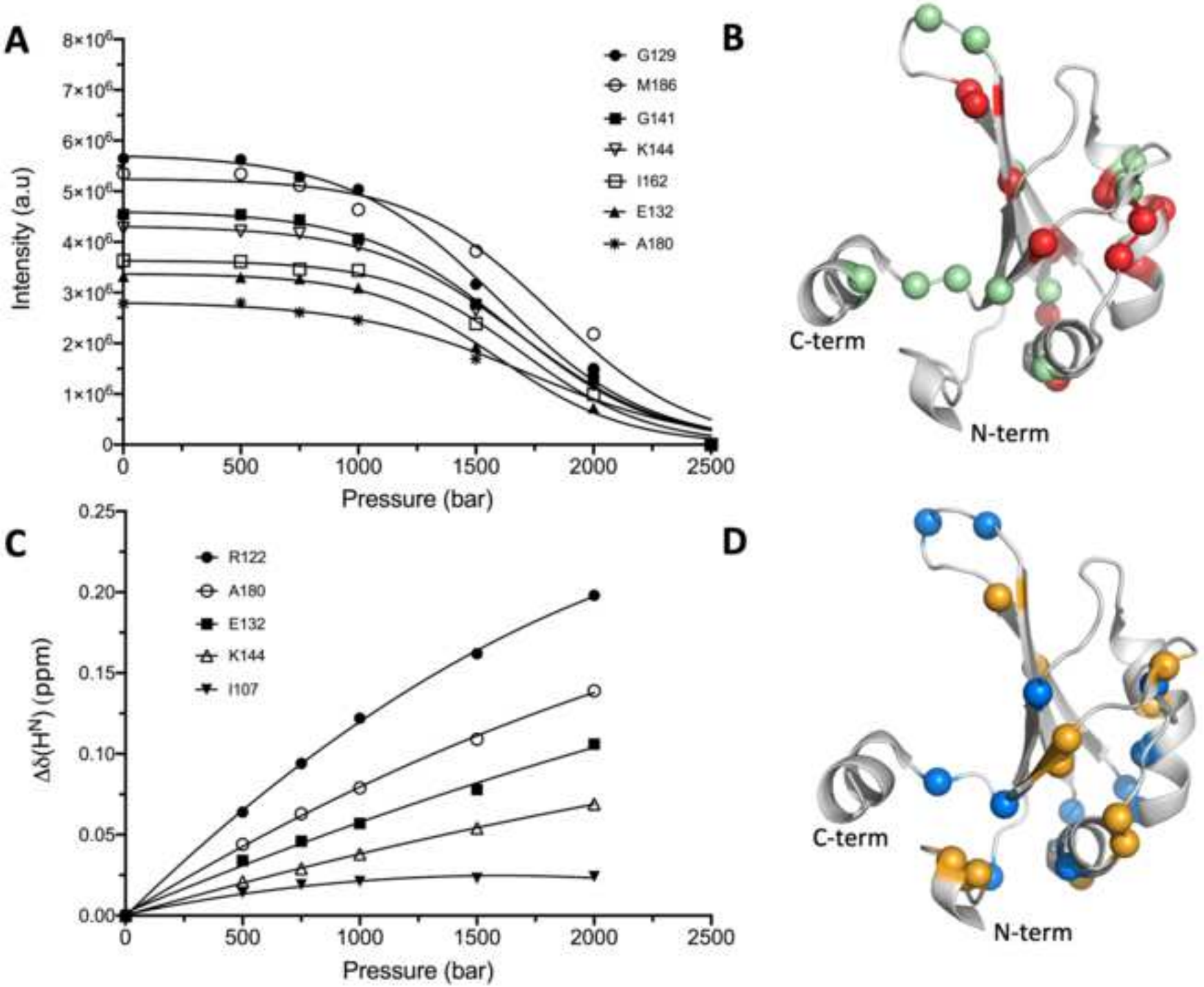

We truly appreciated the comments and insightful suggestions of the reviewers and we revised the manuscript accordingly to clarify all the points mentioned. All changes in the manuscript have been highlighted in yellow.

Editorial comments:
Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proofread the whole manuscript and corrected all the issues we could find.

2. Please revise the following lines to avoid previously published work: 34-35, 62-64, 145-146.

We revised lines 34-35 and 62-64 as follow:

34-35: High-pressure NMR experiments allow a detailed characterization of the factors governing the stability of globular proteins, their folding mechanisms, and oligomerization mechanisms by combining the fine stability tuning ability of pressure perturbation and the site resolution offered by solution NMR spectroscopy.

62-64: The presence of folding intermediates or higher energy states can be detected from large variations in the magnitude of the volume change upon unfolding measured for different residues of a given protein.

Yet, since the lines 145-146 ("With the pressure p in bar and temperature T in kelvin, R = 1.987 cal/K, ΔG_U^0 is in cal/mol and ΔV_U is in cal/mol/bar.") is a technical description of the terms of the equation and we couldn't find a meaningful way to rewrite it.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.

We removed all commercial language from the manuscript.

5. Please ensure that all experimental parameters and details are provided so others can replicate the protocol.
1a) What is the sample size/volume? How much mineral oil is applied? Please quantitate this volume.

We added the following:

1b) Required sample volume is similar to that of a standard 3 mm diameter NMR tube (~300 µl).
1c) Introduce the 15N-labeled sample with a glass pipette into the zirconia tube. Make sure the sample seats at the bottom of the tube. Complete with 200 µl of mineral oil to prevent the sample from mixing with the transmission liquid (e.g. water). Fill the rest of the tube with transmission liquid.

2a/2b) How gradually is the pressure increased/decreased? What is the rate of change?

We changed the paragraph 2a) as follow:

2a) Test the overall stability of the protein by gradually increasing pressure from 1 bar to 2.5 kbar with 500 bar increments. The speed of the pressure pump is set by default at ~18 bar/s. If the precise folding/unfolding rates are not known, it is recommended to let the sample equilibrate 15-20 min after each 500 bar increment. Record a spectrum at 2.5 kbar.

In the software, please ensure that all button clicks and user inputs are provided.

We added the following description in paragraph 3b):

3b) In Sparky menu, click on “peak” > “Peak list (lt)”. In the peak list window, click on “Options” and select the option to display both “Frequencies (ppm”) and “Data height”. Save the list obtained for each spectrum.

6. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We added the following paragraph in the Discussion section:

The whole pressure system (pump and controller) is relatively compact and can be installed on a standard utility cart for easy access to the spectrometer (Fig. 1). All pulse sequences can be used under pressure without modification, including triple resonance experiments. The only limitation is that pressure-rated zirconia tubes reduce the sensitivity of NMR experiments by almost 50% compared to a standard 3 mm diameter NMR tube. This may present a challenge for protein samples that can’t be concentrated to high-enough level. Compared to other perturbation methods such as temperature or chemical denaturants, hydrostatic pressure presents the advantage of being, with very few exceptions, fully reversible. Because pressure favors the dissociation of protein complexes, there is for example very little risk of sample aggregation, a problem often encountered with high temperature.

We also added the following in 3a)

Note: because pressure induces a non-uniform shift of ^{1}H and ^{15}N chemical shifts, the backbone assignment can’t simply be copied from one spectrum to the next one. It has to be manually adjusted.

7. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please provide the titles of the publications.

We modified all the references accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript described a detailed protocol for performing high-pressure NMR in the study of protein structure and dynamics. The authors used three exemplary protein systems, including a well-folded protein, an intrinsically disordered protein, and a protein complex, to showcase how high-pressure NMR can be implemented. The authors also provided an explanation for NMR spectral changes at increasing hydrostatic pressure.

Major Concerns:

No major concern.

Minor Concerns:

Line 96, high pressure can cause protein structural change, manifested as NMR spectral changes. The NMR chemical shift perturbations (CSPs) can be linear or curved. As the CSP pattern can be different for different protein systems, the authors should explain the relationship between structural change and CSP pattern. Line 286, reads "Comparison of the measured chemical shift changes with those calculated from the available database is here crucial to distinguish significant changes from non-specific baseline effect." The

authors should elaborate on how to discard non-specific effects and depict the real changes for the intrinsically disordered protein.

We truly appreciate the reviewer's positive comment. The line 286 emphasizing the importance of comparing chemical shift changes with the available databases refers to the analysis of CSP under pressure for intrinsically disordered proteins. We completely removed this section from the present version of the manuscript and refocused our protocol on the study of well-folded proteins under pressure. Comparison of CSP under pressure with databases is typically not done for well-folded proteins. Yet, we added an additional reference and the following sentence in the discussion to highlight the fact that CSP data can be further analyzed to extract additional thermodynamic information.

It should be noted Kalbitzer and coworkers have demonstrated that a more in-depth analysis of chemical shift changes can be performed linking the ratio between the non-linear and linear coefficients (B_2/B_1) with the ratio of the change in compressibility and volume ($\Delta\beta/\Delta V$).

Reviewer #2:

Manuscript summary:

This is an informative article that will help new practitioners of high-pressure NMR spectroscopy or serve as an educational tool for those curious about the method.

Main comments

1) This reviewer strongly recommends adding a brief mention of baroresistant buffers. Without those, the experiments will yield potentially ambiguous results. The pH (and buffer composition) of the representative results should be included.

We thank the reviewer for this insightful suggestion. We added the following paragraph as 1a)

1a) Choice of buffer: the pKa of anionic buffers such as phosphate and MES is associated with a substantial reaction volume (i.e. the difference in the partial molal volumes of the acid and the ionized products). The pH of such buffers can therefore be significantly affected by a change of pressure (~0.25-0.5 pH unit/kbar). Recommended buffers include equal mixture of anionic and cationic buffers, such as phosphate and Tris.

2) The authors state that the protein is fully unfolded at 2.5 kbar, but this is not consistent with the data in Figure 3A (see, e.g., M186). Is the ~0 value of Iu a fitted parameter? Please clarify.

3) The curves presented in Figure 3A are not well defined. A better case for data analysis would be made if data points were shown every 250 bar between 1 bar and 2.5 kbar, including the data at the high pressure limit.

We apologize for the incomplete description of the fitting procedure and we thank the reviewer for pointing this out. For this specific data set, we don't have data points collected every 250 bar. We initially collected spectra every 500 bar and after visual inspection, we collected another spectrum at 750 bar, near the inflection point of the unfolding transition. Yet, we changed the fitting procedure to make it simpler and hopefully more clear. Instead of having I_U as a fitting parameter, which we agree can be confusing, we rewrote Eq. 1 as follow and added the following clarifications in the Representative Results section:

$$I = \frac{I_F}{1+e^{-[\Delta G_U^0 + \Delta V_U (p-p_0)]/RT}} \qquad \text{Eq. 1}$$

The protocol described here was used to probe the pressure dependence of RRM2, the second RNA recognition motif hnRNPA1 (residues 95-106), which is almost completely unfolded within the 2.5 kbar range (>90%). ^{1}H-^{15}N spectra were collected at 1 bar, 500 bar, 750 bar, 1 kbar, 1.5 kbar, 2 kbar, and 2.5

kbar bar. Since none of the native crosspeaks were visible above the noise level at 2.5 kbar bar, all corresponding residues were attributed an intensity value of 0 at this pressure (Fig. 3A).

Figure 3A shows the fitted intensity profiles with the modified procedure. The results in terms of ΔG and ΔV are virtually identical.

Minor points
Line 44: include references for the biological importance of the sparsely populated states
Lines 45-46: attach one or more relevant references to each of the quoted experiments rather than bunch them at the end of the sentence.
Lines 143-144 and elsewhere: include the word "standard" (1 bar) for the free energy, where appropriate

The manuscript has been modified to address all the minor points listed above.