ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Magnetic Resonance 164 (2003) 84–91

JMR Journal of Magnetic Resonance

www.elsevier.com/locate/jmr

Mixing apparatus for preparing NMR samples under pressure

Wen-Jin Wu, Gediminas Vidugiris,[1] Ed S. Mooberry, William M. Westler, and John L. Markley*

Department of Biochemistry, National Magnetic Resonance Facility at Madison, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA

Received 19 February 2003; revised 17 April 2003

Abstract

The size limit for protein NMR spectroscopy in solution arises in large part from line broadening caused by slow molecular tumbling. One way to alleviate this problem is to increase the effective tumbling rate by reducing the viscosity of the solvent. Because proteins generally require an aqueous environment to remain folded, one approach has been to encapsulate hydrated proteins in reverse micelles formed by a detergent and to dissolve the encapsulated protein in a low-viscosity fluid. The high volatility of suitable low-viscosity fluids requires that the samples be prepared and maintained under pressure. We describe a novel apparatus used for the preparation of such samples. The apparatus includes a chamber for mixing the detergent with the low-viscosity solvent, a second chamber for mixing this with hydrated protein, and a 5-mm (o.d.) zirconium oxide NMR sample tube with shut-off valves designed to contain pressures on the order of 10 bar, sufficient for liquid propane. Liquids are moved from one location to another by introducing minor pressure differentials between two pressurization vessels. We discuss the operation of this apparatus and illustrate this with data on a 30-kDa protein complex (chymotrypsin:turkey ovomucoid third domain) encapsulated in reverse micelles of the detergent, sodium bis (2-ethylhexyl) sulfosuccinate, aerosol-ot (AOT), dissolved in liquid propane.
© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Apparatus, pressure; Propane, liquid; Pressure; Protein; Reverse-micelle

1. Introduction

Over the past decade, triple resonance ^{1}H, ^{15}N, and ^{13}C spectroscopy has provided a powerful methodology for resonance assignments of proteins labeled uniformly with ^{13}C and ^{15}N [1,2], and complete sequence assignments of proteins up to 25 kDa are now routine. NMR spectroscopy of larger proteins generally requires that the signals be sharpened or simplified through the use of a more complicated labeling pattern, such as extensive deuteration [3], segmental labeling, or selective labeling [4–6]. A limitation of this approach is that NOEs important for defining the structure of the protein frequently are eliminated.

Transverse relaxation-optimized spectroscopy (TROSY) is a method that exploits constructive interference between relaxation caused by dipole–dipole interactions and chemical shift anisotropy [7]. This technique has increased the sensitivity and resolution of NMR experiments for large proteins by several-fold. Several of TROSY-based triple resonance experiments for sequential NMR assignments of large proteins are described in the literature [8–10]. The cross-correlated relaxation-induced polarization transfer (CRIPT) technique designed for very large molecules or complexes (>200 kDa) results in a two to threefold gain in sensitivity in addition to the sensitivity gain due to TROSY [11,12]. TROSY and CRIPT methods can be combined with the high-level and selective deuteration experiments described above to open up a new avenue for solution NMR spectroscopy of large proteins. Very recently, NMR analysis of a 900-K complex of GroEL–GroES has been reported [13]. The essential requirement for TROSY effects is a high-magnetic field strength: 500–800 MHz is optimal for T_2

* Corresponding author. Fax: +608-262-3759.
E-mail address: markley@nmrfam.wisc.edu (J.L. Markley).
[1] Present address: Promega Corporation, 2800 Woods Hollow Rd., Madison, WI 53711, USA.

1090-7807/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1090-7807(03)00144-7

suppression in ^{1}H–^{13}C correlations and 1 GHz is optimal for T_2 suppression in ^{1}H–^{15}N correlations [14].

Another promising, and entirely complementary, approach to NMR spectroscopy of large proteins is to narrow linewidths by lowering the viscosity of the solution. Although the viscosity of an aqueous solution can be lowered by increasing the temperature, for proteins this method is limited severely by thermal denaturation of the sample. A more effective approach is to encapsulate each hydrated protein molecule in a reverse micelle and to dissolve the micelle in a low-viscosity apolar solvent, such as butane or propane [15] or supercritical carbon dioxide [16]. Wand and coworkers demonstrated high-resolution NMR spectra of ubiquitin encapsulated in reverse micelles dissolved in low-viscosity solvents and showed that the protein linewidths scaled with the viscosity of the solvent [15]. They also published the design of a custom pressurized NMR cell assembly and mixing apparatus for the preparation of proteins encapsulated in reverse micelles dissolved in low-viscosity solvents [17]. Wand and coworkers further found that the structure of ubiquitin in reverse micelles was essentially identical to that in aqueous solution and in crystals [18]. These results are summarized in a recent review [19]. Independently, Bax and coworkers demonstrated high-resolution NMR spectra of detergent-encapsulated bovine pancreatic trypsin inhibitor (BPTI) dissolved in low-viscosity liquid CO_2 [16].

Reverse micelles are thermodynamically stable water droplets surrounded by a monolayer of surfactant molecule dispersed in a low-polarity solvent. Spontaneous formation of the micelles occurs with gentle agitation of the solution. A vast literature describes the field of reverse micelles and numerous reviews discuss proteins encapsulated in reverse micelles [19–24]. The most widely studied surfactant for the formation of reverse micelles is the sodium salt of bis (2-ethylhexyl) sulfosuccinate, abbreviated "AOT" (aerosol-ot). Its advantage stems from the fact that the reverse-micelle phase covers a large region of the AOT/water/hydrocarbon phase diagram [25]. By incorporating biomolecules into reverse micelles, an aqueous environment is maintained for the molecule, while the micelle is soluble in a non-polar solvent. When the molar ratio of water to AOT is about 10, the radius of a reverse micelle encapsulating a protein is about 15 Å larger than that for the aqueous protein itself. This additional size comes mostly from the tails of the surfactant molecule and the hydration layer around the protein. Thus the viscosity of the non-polar solvent must be considerably lower than that of water for the tumbling rate of the encapsulated protein to be faster than that of the free protein.

A short-chain alkane, such as propane, has the required low-viscosity needed to substantially reduce the correlation time of an encapsulated protein. However, the sample must be maintained under pressure to keep the solvent in the liquid state. The pressures required (under 10 bar) are two orders of magnitude lower than those that lead to protein denaturation. Wand and coworkers have discussed the requirements of apparatus for mixing samples and collecting NMR data under pressure [17]. Owing to the limited solubility of protein encapsulated in reverse micelles, they designed a pressurizable NMR tube with a relatively large volume (8 mm o.d./5 mm i.d.). Recent advances in NMR probe technology have produced triple resonance cryogenic probes that offer as high as fourfold increase in sensitivity as compared to conventional probes. We report here the design and operation of a general-purpose apparatus for preparing proteins encapsulated in reverse micelles under pressure and for transferring them to 5 mm o.d. NMR tubes for data collection.

2. Materials and methods

All stainless steel valves and tubing were purchased from the High Pressure Equipment Company. Polyetheretherketone (PEEK) polymer fittings, perfluoro alkoxy alkane (PFA) Teflon tubing, and metering valves were purchased from Upchurch Scientific. The mixing cells were manufactured by the University of Wisconsin-Madison Physics Instrument Shop; fused silica windows were purchased from Janos Technology. Zirconium oxide NMR tubes were manufactured by O'Keefe Ceramics (Woodland Park, CO). Aluminum breadboards, stainless steel mounting posts, and post holders were purchased from ThorLabs. A Rubbermaid flat-shelf service cart was purchased from Global Industrial Equipment. Sodium bis (2-ethylhexyl) sulfosuccinate (AOT) and 2-methylbutane (isopentane) were purchased from Sigma. Liquid propane, high-purity nitrogen gas and a 207-bar (3000 psi) pressure check valve were purchased from Matheson Tri-Gas. Deuterated isopentane was from Isotech; deuterated propane (d_8) gas was from Cambridge Isotope Laboratories.

Three types of tubing were used: stainless steel, PEEK, and Teflon PFA (perfluoroalkoxy). PEEK tubing was used at the points where flexibility was needed. Translucent Teflon PFA tubing was used at the points where liquid viewing was needed. The vapor pressure of liquid propane is 8.48 bar at 21.1 °C. The pressure ratings for all tubing sections were well above this number.

2.1. Test of solution condition for the encapsulated protein in reverse micelles

Solution conditions for individual proteins were investigated by preparing encapsulated proteins at various protein concentrations and protein/water ratios in li-

quid isopentane, because these trials can be carried out at atmospheric pressure. The criteria used in judging the results were lack of precipitation and concentration of protein in solution. The optimal conditions identified in these trials were then used in the apparatus described here for preparing AOT encapsulated hydrated proteins dissolved liquid propane. Dry protein powder in an Eppendorf tube was hydrated with the desired amount of water containing buffer salt and then mixed with AOT in isopentane solution. Mixing was achieved by manual shaking of the Eppendorf tube. The solution was then centrifuged to remove any precipitate. The concentration of the protein in reverse micelles was measured by reading its absorbance at 280 nm on a Hewlett Packard 8452A diode array spectrophotometer. An AOT/isopentane solution was used as the blank. The extinction coefficient of the encapsulated protein was assumed to be comparable to that in water. Owing to the volatile nature of isopentane, closed screw cap UV quartz cuvettes were used for absorbance measurements.

3. Results and discussion

3.1. Overall design

A schematic diagram of the mixing apparatus is shown in Fig. 1. The main components consist of a loop of tubing that meters out the volume of liquid propane to be dispensed (L2), a surfactant-mixing cell (C1), a fixed-volume tubing section (L3) used to deliver a given volume of surfactant solution, a protein mixing cell (C2), and a custom NMR cell assembly with shut-off valves. The system is pressurized by nitrogen gas. Two stainless steel cylinders, each with 656-ml internal volume, were used as pressure reservoirs on each end of the system. A small pressure differential between these reservoirs served to transfer liquids from one part of the system to another with appropriate opening and closing of valves. The internal volumes of the reservoirs were large compared to those of the tubing and mixing cells; no substantial pressure alteration occurred when valves were opened. The mixing apparatus was mounted on a service cart for mobility. This permitted the system to be

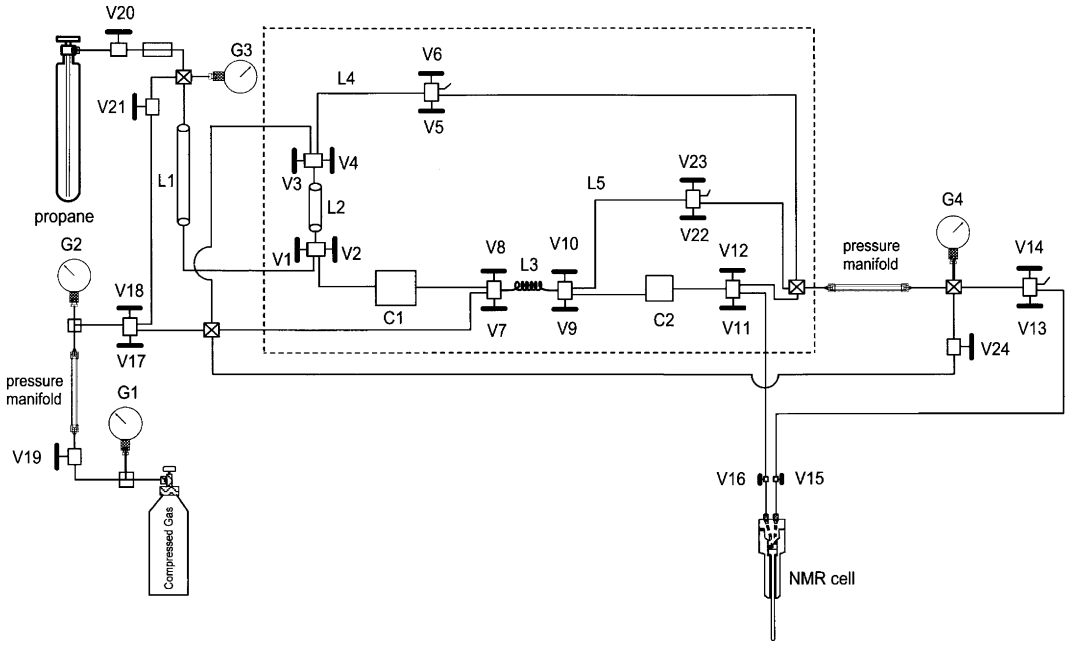

Fig. 1. Schematic diagram of the mixing apparatus used to prepare proteins in reverse micelles under pressure. *Tubing*: L1, PFA Teflon loop (internal volume ~10 ml); L2, PFA Teflon loop (internal volume 3.09 ml); L3, PFA Teflon loop (600 μl internal volume 1/8″ o.d.); L4 and L5, PFA Teflon loop (1/16″ o.d.), for liquid viewing purpose. *Pressure cell*: C1, surfactant mixing pressure cell (4.9 ml internal volume) (see Fig. 2 for details); C2, protein mixing cell (1.1 ml internal volume) (see Fig. 2 for details). *Valves*: stainless steel valves are labeled as V# for each stem, valve 15 and valve 16 are micro-metering valves with their own shut-off thumbscrew; with proper arrangement (one higher than the other), these micro-metering valves are small enough to fit in the standard NMR magnet. *Pressure manifold*: Stainless steel pressure reservoir (656 ml internal volume). *Pressure gauge:* labeled as "G". *NMR cell assembly:* detachable NMR cell assembly, see Appendix A for details. The small rectangular box at the right-hand side of V20 represents a pressure check valve (200 bar) that prevents accidental transfer of nitrogen gas from the system to the liquid propane tank. The apparatus within the dashed line is mounted on a separate breadboard, which is connected to the larger breadboard by a vertical stainless steel post. Each of the mixing chambers is mounted on a hinged breadboard (ThorLabs). This design allows the dashed portion of the mixing apparatus to be rotated. A 90° rotation of either mixing cell (C1 or C2) changes the orientation of the outlet from the side (for mixing) to the bottom (for dispensing liquid). The mixing apparatus, with the exception of the liquid propane and nitrogen gas tanks, is mounted on a large aluminum breadboard that is fastened to a flat-shelf service cart.

transferred to a cold room for preparation of samples that required low temperatures.

3.2. Design of the mixing cell

The design of the pressure cells was modified from that of Betts and Bright [26]. The cell body was made of 316-stainless steel with two exits drilled to accept HPLC fittings. The two mixing cells were of the same overall design except for the volume. The surfactant-mixing cell (Fig. 2) had an internal volume of 4.9 ml (useful volume of 4.15 ml below the inlet and outlet ports); the protein-mixing cell had an internal volume of 1.1 ml (0.71 ml below the ports). The surfactant-mixing cell was designed to prepare sufficient AOT–propane mixtures for multiple sample preparations. The inlet and outlet of the mixing cell are mutually perpendicular. The cell stands vertically during the liquid intake. With a 90° rotation, the outlet then faces downward for liquid transfer out of the cell. Each end of the pressure cell was sealed with a fused silica window sandwiched by two Teflon washers. A hollow stainless steel screw cap was used to seal the cell at each side.

3.3. NMR cell

The designs of the NMR tube holders (“spinners”) for standard Bruker and Varian 5 mm probes and of the zirconium oxide 5 mm o.d./3.3 mm i.d. NMR tube were adapted from the 8 mm o.d./5 mm i.d. design of Wand and coworkers [17]. Details are provided in Appendix A.

The material used for the spinner valve was 7075-aluminum, instead of BeCu used previously [17]. The use of aluminum eliminates any health hazards from the

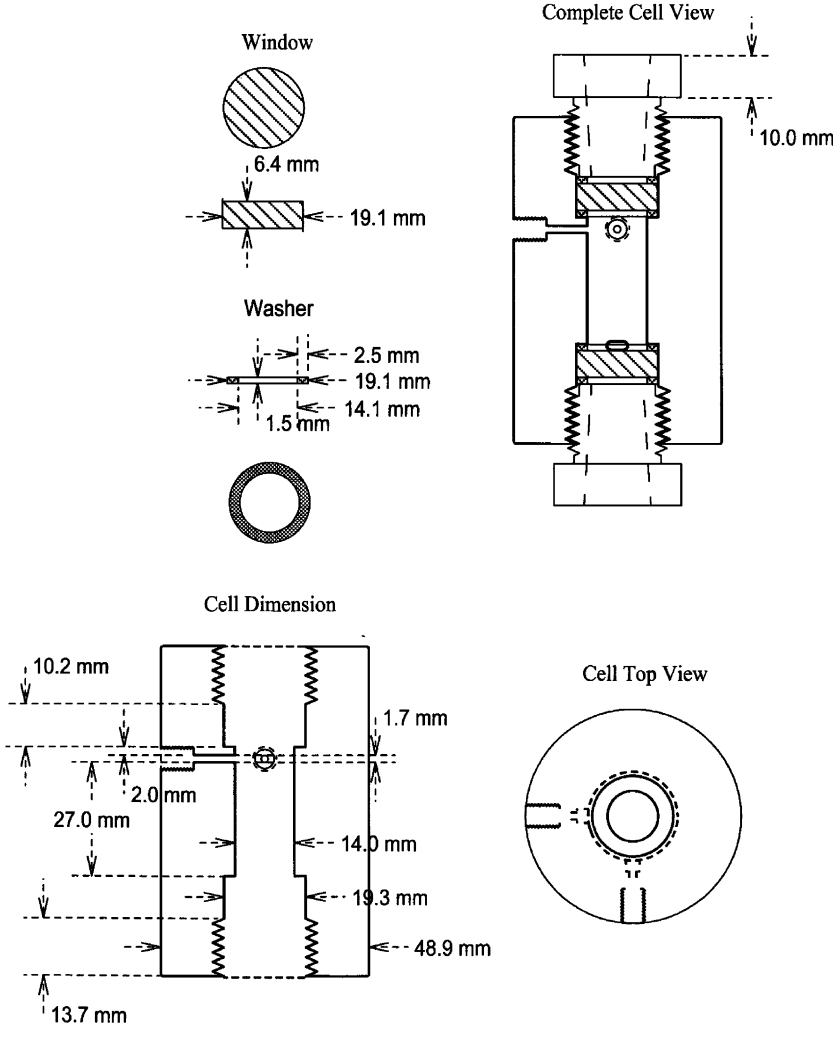

Fig. 2. Schematic diagram for the surfactant-mixing cell. The cell, which is constructed of 316-stainless steel with two exits drilled to accept two HPLC fittings, is sealed at each end with a fused silica window sandwiched by two Teflon washers. A hollow stainless steel screw cap can be screwed in to seal the cell at each side. The internal volume of the pressure cell is 4.9 ml (4.15 ml below the I/O ports). The fused silica window is 19.1 mm in diameter and 6.4 mm in thickness. The inlet and outlet of the cell are mutually perpendicular. The cell stands vertically during the liquid transfer into the cell. This perpendicular arrangement prevents liquid from spilling out during filling. With a 90° rotation, the outlet can be pointed downward for liquid transfer.

BeCu material. The valve was machined to accept two 10/32 HPLC fittings. The NMR tube was sealed to the collar by the action of an embedded o-ring (B and C). Wand's group used an o-ring at low pressure and a boron nitride washer at high pressure to provide the seal at position C. We also found that an o-ring in position C provided a much better seal for liquid propane than a boron nitride washer. We used two PEEK micro-metering valves (55 bar pressure rating) to seal off the top of the cell assembly. After transferring the solution containing reverse-micelle encapsulated protein into the NMR cell assembly, the metering valves were shut off and detached from the mixing apparatus along with the NMR cell assembly. The open end of the metering valve was closed by a PEEK plug, which was sanded down to a dimension allowing the whole unit to fit into the warm bore of the NMR magnet.

3.4. Safety issues

Although we never experienced any problem that posed a threat to safety, it should be borne in mind that pressurized NMR samples present a potential hazard. Safety glasses and protective clothing should be worn, and normal precautions taken. During filling and transportation, pressurized NMR tubes were kept in transparent plastic cylinders. To check for leakage, pressurized tubes were kept in a protective cylinder in a fume hood for at least 1 h prior to moving them to the NMR spectrometer. The pressurized NMR tubes were lowered carefully into NMR probe to avoid breakage. Extreme care was taken to prevent uncontrolled heating of samples by the variable temperature unit or radio-frequency decoupler.

4. Detailed operation of the mixing apparatus

The encapsulation of hydrated proteins into AOT-reversed micelles dissolved in apolar low-viscosity solvent consists of three major steps: (1) liquefaction of the low-viscosity hydrocarbon solvent, (2) solvation of the AOT in the solvent, and (3) transfer or distribution of the hydrated protein into the AOT-solvent phase via encapsulation.

4.1. Pre-transfer of liquid propane

Our design introduces a simple liquid transfer mechanism that avoids the use of expensive devices such as a high-pressure syringe needle valve. The propane can be introduced as a liquid; alternatively, it can be introduced as a gas into the proximal pressurization cylinder and then liquefied by pressurization of that cylinder with nitrogen gas. Visual inspection of the clear L1 loop fabricated of PFA tubing indicates whether or not liquid is present. Transfer of liquid propane from the bulk liquid propane tank into the L1 loop is achieved by using a pressure differential of 1.4–2.1 bar between the two pressure reservoirs. The internal pressure of the propane tank is 7.6 bar, and the pressure in the propane transfer path (V20–L1–V1–L2–V4–V5–pressure bomb) is set to 5.5–6.2 bar.

4.2. Fixed-volume liquid propane transfer

The L2 loop meters the amount of liquid propane introduced into the first mixing chamber. The L2 loop, also fabricated from clear PFA tubing, is filled from the bottom to the top of the loop with the L2 loop standing vertically by transfer of liquid propane from the L1 loop. The vertical orientation of the tube allows removal of any gas bubbles. Unless otherwise indicated, this and all other liquid transfers utilize a pressure differential of $\sim$0.7 bar. During the filling, liquid propane flows from the L1 loop, through V1, and into the L2 loop. The filling is known to be complete when excess liquid propane flows into the clear L4 loop. The fixed volume of liquid propane in L2 is then transferred by pressure difference into the C1 surfactant-mixing cell, which has been preloaded with a known mass of solid AOT and a magnetic stir-bar. Valve 3 serves as a switch to provide nitrogen gas pressure for this liquid transfer.

4.3. AOT/propane solution

The contents of C1 are mixed by magnetic stirring until a homogeneous and transparent solution of AOT in liquid propane is achieved. Our design, which permits accurate measurement of the liquid propane volume prior to mixing, provides superior control over the composition of the resulting mixture than the previous design in which propane is liquefied directly within a pressure cell in the presence of AOT [15]. This is important because the size of a reverse micelle depends on the molar ratio of water to surfactant.

4.4. Distribution of fixed volume AOT solution

The C1 mixing chamber permits preparation of a 3-ml stock solution of AOT in propane, enough for multiple samples. The length of the surfactant loop L3 accurately determines the volume of this solution, 600 μl in the present case, to be dispensed from the C1 cell. The following steps are performed in filling the surfactant loop. The rotary frame, highlighted by the dashed line in Fig. 1, is rotated by 90° so that the outlet of the surfactant cell faces downward. A very small pressure difference ($\sim$0.07 bar) introduced between the C1 cell and the surfactant loop leads to filling of the surfactant loop, which is complete once a small excess of AOT solution

flows into the liquid viewing loop after V10. The small pressure difference prevents the formation of bubbles which would introduce error into the volume being metered and allows the liquid to flow slowly so the valve can be closed as soon as the loop is filled. This avoids undesired overflow of AOT solution into the liquid viewing loop and the concomitant loss of solvent, which may contain an expensive lock compound such as deuterated liquid propane.

4.5. Encapsulation of protein into reverse micelles and transfer to the NMR cell

The AOT solution in the surfactant loop is subsequently transferred into the protein-mixing cell, which has been preloaded with hydrated protein and a magnetic stir-bar. In our experience, the small stir-bar needs to be in motion before the AOT/propane solution is transferred so as to avoid the formation of a precipitate which may be difficult to dissolve. The mixture is stirred until the encapsulation process is complete. The resulting solution is then transferred into a ZrO_2 NMR cell assembly by a pressure difference of 0.3–0.7 bar. In our design, commercially available metering valves (55 bar, Upchurch Scientific) are used in connecting the NMR cell assembly and the mixing apparatus. The metering valve contains its own thumbscrew shut-off nut and is small enough to fit into a standard bore magnet. The NMR cell assembly is lowered into the magnet by a string. An audible click occurs when the sample is lowered into the correct position. NMR spectra were acquired without spinning.

4.6. Addition of deuterated solvent as lock signals

Wand and coworkers reported the use of a capillary containing D_2O as the lock solvent. The capillary was placed directly inside an 8 mm o.d./5 mm i.d. zirconium oxide NMR tube along with the protein reverse micelle solution. To the avoid loss of filling factor in a 5 mm o.d./3.3 mm i.d. NMR tube, we use internal deuterated solvents (isopentane or propane) to provide the lock signal. A mixture of AOT/10% isopentane-d_{12}/90% propane provides a transparent single-phased solution. In using deuterated propane as the lock solvent, propane-d_8 gas was first introduced into the proximal 656 ml pressure reservoir; the pressure was then raised to 2.1–2.8 bar above the liquefaction pressure. The desired volume of liquid propane-d_8 (20% of the final total volume) was transferred to the surfactant-mixing cell that contained AOT in a defined volume of propane. Very recently, Wand's group reported the use of 20% deuterated *n*-pentane as the lock solvent for protein reverse micelles dissolved in *n*-pentane and in liquid propane [27].

4.7. Proteins in reverse micelles

We have used the mixing apparatus to prepare encapsulated proteins in AOT-reverse micelles dissolved in liquid propane. As a model system, we have investigated turkey ovomucoid third domain (OMTKY3, a 6-kDa proteinase inhibitor) complexed to the serine proteinase chymotrypsin (24 kDa). The association constant for complex formation is 1.8×10^{11} [28]. ^{15}N-Labeling of the small inhibitor simplified the spectra, and allowed us to collect spectra in water and in reverse micelles quickly. Fig. 3 compares ^{1}H–^{15}N-HSQC spectra of the complex between [^{15}N]OMTKY3 and chymotrypsin in aqueous solution and in reverse micelles. The close correspondence between the two spectra indicates that the structure of OMTKY3 in the complex encapsulated in reverse micelles is similar to that in the complex in aqueous solution. Although it was expected that the linewidths

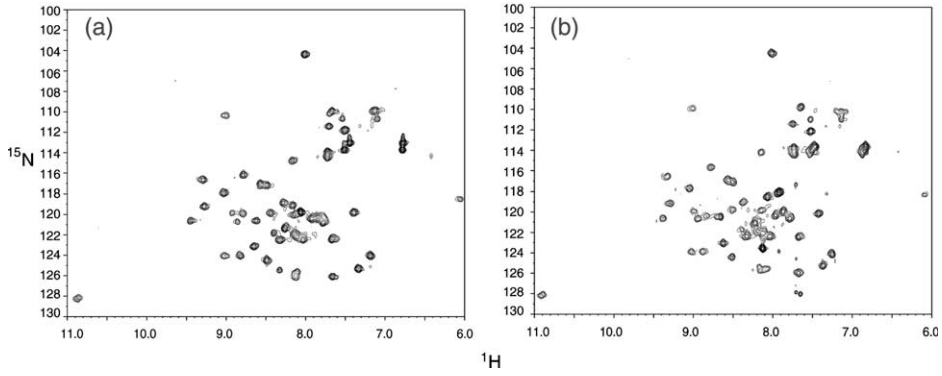

Fig. 3. ^{1}H–^{15}N-HSQC spectra of [^{15}N]OMTKY3:chymotrypsin (a) in free aqueous buffer and (b) in AOT reverse micelles dissolved in liquid propane. In both cases, the protein was solvated by aqueous buffer containing 50 mM Tris-d_{11}, pH 7.2. The reverse micelle sample was under 15 bar pressure in a 5-mm o.d. zirconium oxide tube. Gradient enhancement-type HSQC experiments were acquired. Both spectra were acquired at 298K on a Bruker DMX 600 spectrometer equipped with triple axis gradients and a triple resonance probe. The spectral widths were 8090 Hz (1024 complex points) in the ^{1}H dimension and 2433 Hz in the ^{15}N dimension (128 complex points). The protein concentration was approximately 0.4 mM in the free aqueous sample and was approximately 0.2 mM in the reverse micelle sample.

from the encapsulated protein would be sharper than those from the complex in aqueous solution, they were similar under the two conditions. A number of possible explanations for this result need to be explored. The reverse micelles could each contain two encapsulated protein complexes; this question can be addressed by NMR measurements of the lateral diffusion coefficient. Contamination of the surfactant with water could have led to an erroneous estimate of the water to surfactant ratio. In addition, viscosity of the solution may have been affected by the loss of about 30% of the solution volume during the transfer steps. This occurred presumably through diffusion of gaseous propane within the system; this can be minimized in the future by pre-equilibration of the pressurized system with propane.

During the preparation of this manuscript, Wand's group reported a new 5 mm o.d. pressurizable NMR tube made of PEEK along with a simplified design for the NMR cell assembly [27]. This new type of NMR cell assembly could be connected to our mixing apparatus by means of a stem valve.

Acknowledgments

We thank Dr. Jikui Song for the assistance in preparing the [^{15}N]OMTKY3:chymotrypsin complex. NMR studies were carried out at the National Magnetic Resonance Facility at Madison with support from the NIH Biomedical Technology Program (RR02301). Additional equipment funding came from the University of Wisconsin, NSF Academic Infrastructure Program (BIR-9214394), NIH Shared Instrumentation Program (RR02781, RR08438), NIH Research Collaborations to Provide 900 MHz NMR Spectroscopy (GM66326), NSF Biological Instrumentation Program (DMB-8415048), and the US Department of Agriculture.

Appendix A

Fig. 4 is a schematic showing designs for the NMR cell assembly to fit a standard Bruker 5 mm probe and standard Bruker high-resolution shim stack and for the 5 mm o.d. NMR zirconium oxide tube (middle). The custom manufactured valve is shown at the right-hand side of the drawing. Components are lettered: A, spinner

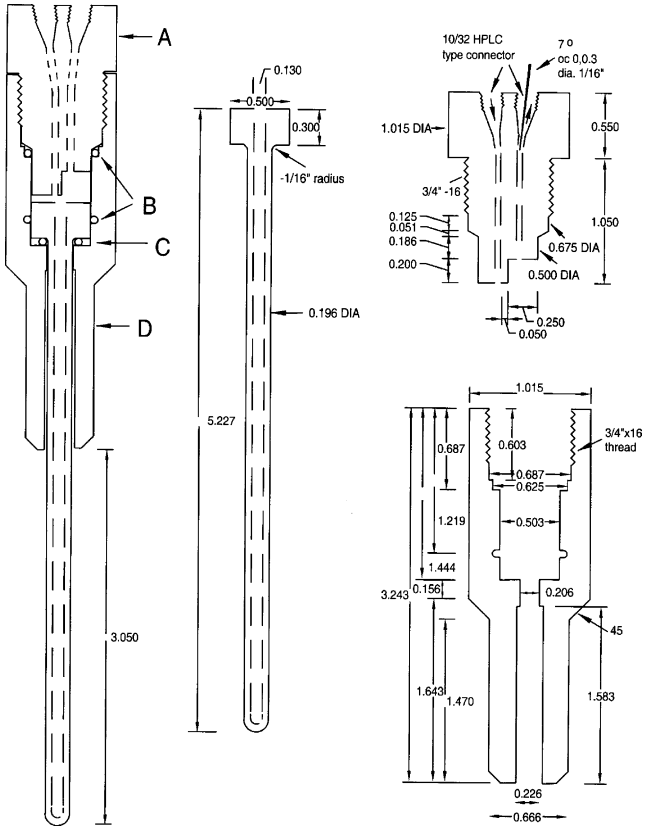

Fig. 4.

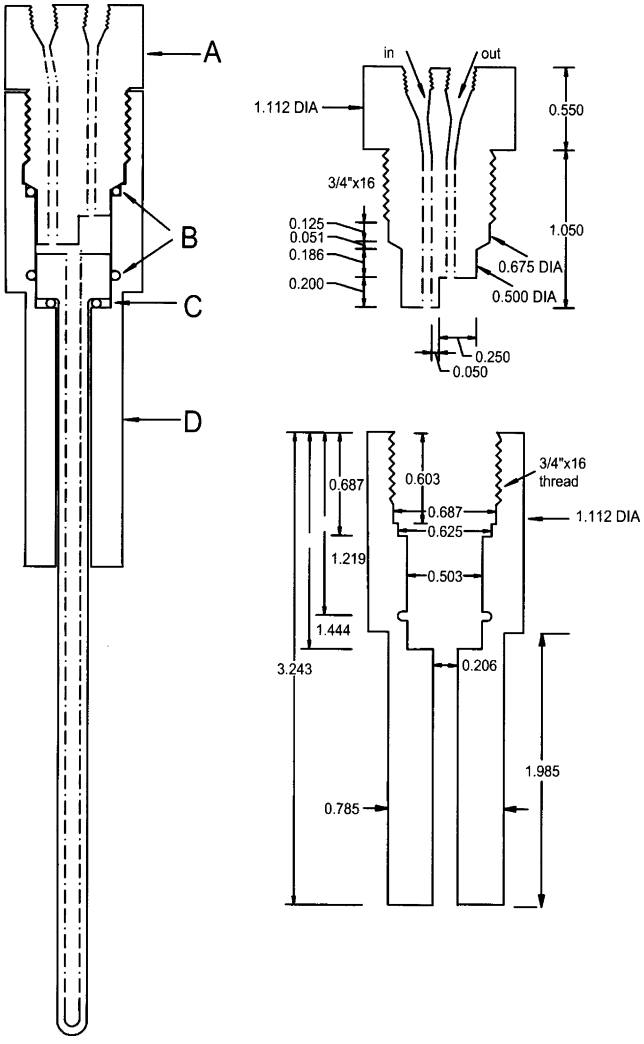

Fig. 5.

valve cap; B, rubber o-ring; C, rubber o-ring at low pressure or boron nitride washer at high pressure; and D, spinner valve. The spinner valves (A and D) were made of 7075-aluminum. We found that a regular o-ring at position C provides a better seal than a boron nitride washer at low pressure (21 bar). All units are in inches.

Fig. 5 shows the design for the NMR cell assembly for use with a standard Varian 5 mm probe and high-resolution shim stack. The only difference between this and that shown in Fig. 1 is the dimension of the spinner valve. They both accommodate the same zirconium oxide NMR tube shown in Fig. 1. The sample depth is 65 mm in a standard Varian 5 mm probe. All units are in inch.

References

[1] G.M. Clore, A.M. Groneborn, Structure of larger proteins in solution: three- and four-dimensional heteronuclear NMR spectroscopy, Science 252 (1991) 1390–1399.

[2] A. Bax, Multidimensional nuclear magnetic resonance methods for protein studies, Curr. Opin. Struct. Biol. 4 (1994) 738–744.

[3] K.H. Gardner, L.E. Kay, The use of ^{2}H, ^{13}C, ^{15}N multidimensional NMR to study the structure and dynamics of proteins, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 357–406.

[4] R. Xu, B. Ayers, D. Cowburn, T.W. Muir, Chemical ligation of folded recombinant proteins: segmental isotopic labeling of domains for NMR studies, Proc. Natl. Acad. Sci. USA 96 (1999) 388–393.

[5] D. Cowburn, T.W. Muir, Segmental isotopic labeling using expressed protein ligation, Method Enzymol. 339 (2001) 41–54.

[6] T. Otomo, K. Teruya, K. Uegaki, T. Yamazaki, Y. Kyogoku, Improved segmental isotope labeling of proteins and application to a larger protein, J. Biolmol. NMR 14 (1999) 105–114.

[7] K. Pervushin, R. Riek, G. Wider, K. Wüthrich, Attenuated T2 relaxation by mutual cancellation of dipolar–dipolar coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, Proc. Natl. Acad. Sci. USA 94 (1997) 12366–12370.

[8] M. Salzmann, G. Wider, K. Pervushin, H. Senn, K. Wüthrich, TROSY-type triple-resonance experiments for sequential NMR assignments of large proteins, J. Am. Chem. Soc 121 (1999) 844.

[9] M. Salzmann, K. Pervushin, G. Wider, H. Senn, K. Wüthrich, NMR assignment and secondary structure determination of an octameric 110 kDa protein using TROSY in triple resonance experiments, J. Am. Chem. Soc 122 (2000) 7543–7548.

[10] D. Yang, R.A. Venters, G.A. Mueller, W.Y. Choy, L.E. Kay, TROSY-based HNCO pulse sequences for the measurement of ^{1}HN-^{15}N, ^{15}N-^{13}CO, ^{1}HN-^{13}CO, ^{13}CO-$^{13}C^{\alpha}$ and ^{1}HN-$^{13}C^{\alpha}$ dipolar coupling in ^{15}N, ^{13}C, ^{2}H-labeled proteins, J. Biolmol. NMR 14 (1999) 333–343.

[11] R. Riek, G. Wider, K. Pervushin, K. Wüthrich, Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules, Proc. Natl. Acad. Sci. USA 96 (1999) 4918–4923.

[12] G. Wider, K. Wüthrich, NMR spectroscopy of large molecules and multimolecular assemblies in solution, Curr. Opin. Struct. Biol. 9 (1999) 594–601.

[13] J. Fiaux, E.B. Bertelsen, A.L. Horwich, K. Wüthrich, NMR analysis of a 900K GroEL–GroES complex, Nature 418 (2002) 207–211.

[14] K. Pervushin, R. Riek, G. Wider, K. Wüthrich, Transverse relaxation-optimized spectroscopy (TROSY) for NMR studies of aromatic spin systems in ^{13}C-labeled proteins, J. Am. Chem. Soc 120 (1998) 6394–6400.

[15] A.J. Wand, M.R. Ehrhardt, P.F. Flynn, High-resolution NMR of encapsulated proteins dissolved in low viscosity fluids, Proc. Natl. Acad. Sci. USA 95 (1998) 15303–15308.

[16] S. Gaemers, C.J. Elsevier, A. Bax, NMR of biomolecules in low viscosity, liquid CO2, Chem. Phys. Lett. 301 (1999) 138–144.

[17] M.R. Ehrhardt, P.F. Flynn, A.J. Wand, Preparation of encapsulated proteins dissolved in low viscosity fluids, J. Biolmol. NMR 14 (1999) 75–78.

[18] C.R. Babu, P.F. Flynn, A.J. Wand, Validation of protein structure from preparation of encapsulated proteins dissolved in low viscosity fluids, J. Am. Chem. Soc 123 (2001) 2691–2692.

[19] P.F. Flynn, A.J. Wand, High-resolution nuclear magnetic resonance of encapsulated proteins dissolved in low viscosity fluids, Method Enzymol. 339 (2002) 54–70.

[20] P.L. Luisi, L.J. Magid, Solubilization of enzymes and nucleic acids in hydrocarbon micellar solutions, CRC Crit. Rev. Biochem. 20 (1986) 409–474.

[21] M.P. Pileni, Structures and Reactivity in Reverse Micelles, Elsevier Press, Amsterdam/Oxford/New York/Tokyo, 1989.

[22] C. Nicot, M. Waks, Proteins as invited guests of reverse micelles: conformational effects, significance, applications, Biotechnol. Genet. Eng. Rev. 13 (1993) 267–314.

[23] T.K. De, A. Maitra, Solution behavior of aerosol ot in non-polar solvents, Adv. Colloid Interf. Sci. 59 (1995) 95–193.

[24] M. Tuena de Gómez-Puyou, A. Gómez-Puyou, Enzymes in low water systems, Crit. Rev. Biochem. Mol. Biol. 33 (1998) 53–89.

[25] H. Kunieda, K. Shinoda, Phase diagram of water-aot-isooctane, Colloid Interf. Sci. 70 (1979) 577–583.

[26] T.A. Betts, F.V. Bright, Instrumentation for steady-state and dynamic fluorescence and absorbance studies in supercritical media, Appl. Spectrosc. 44 (1990) 1196–1202.

[27] P.F. Flynn, M.J. Milton, C.R. Babu, A.J. Wand, A simple and effective NMR cell for studies of encapsulated proteins dissolved in low viscosity solvents, J. Biolmol. NMR 23 (2002) 311–316.

[28] M.J. Laskowski, M. Tashiro, M.W. Empie, S.J. Park, I. Kato, W. Ardelt, M. Wieczorek, in: N. Katanuma, H. Umezawa, M. Holzer (Eds.), Proteinase Inhibitors: Medical and Biological Aspects, Springer, Berlin, 1983, pp. 55–68.