Toward Structural Biology in Supercooled Water

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Internal mobility limits the accuracy of NMR[1] structures:[2] NOEs are quenched, and conformational and/or chemical exchange broaden resonances, thus impeding extraction of conformational constraints. A shift of temperature, *T*, may move such processes into regimes of very fast or slow exchange on the chemical shift time scale. While a large *increase* of *T* is limited by macromolecular stability and excitation of yet additional motions, a *decrease* well below 0 °C is attainable in supercooled water.[3] This promises more accurate NMR structures, a means to freeze out conformations and novel insights into biomolecular dynamics, hydration, and cold denaturation. NMR of small carbohydrates allowed observation of hydroxyl protons,[4] but multidimensional spectra of macromolecules have not been reported. Here we show the feasibility of NMR-based structural biology in supercooled water.

NMR in supercooled water is hampered by high viscosity, η, yielding long overall rotational correlation times, τ_c, and line broadening; an exponential, $\eta(T)$, was fitted to published values[3] (Figure 1a). Hydrodynamic theory[5] predicts for rigid spherical proteins that $\tau_c = 4\pi[\eta(T)]r_H^3/3kT$ (eq 1). r_H is the effective radius with $r_H = [3VM/(4\pi N_A)]^{1/3} + r_w$ (eq 2), where $\bar{V} = 0.73$ cm^3/g, M, N_A and r_w are the protein's specific volume and molecular weight, Avogadro's number, and the added radius of a monolayer of water, respectively. With $r_w = 3.2$ Å, eq 2 yields $r_H = 17.2$ Å for 9.4 kDa recombinant ubiquitin.[6] To verify that theory applies at <0 °C, we determined τ_c between 25 and −15 °C from ^{15}N $T_1/T_{1\rho}$ ratios[7,8] (Figure 1b; Table S1). With $\eta(T)$ of Figure 1a, a fit of eq 1 to τ_c yields $r_H = 17.2 \pm 1.0$ Å and allows prediction of τ_c below −15 °C (Figure 1b). The very good agreement between theory and experiment suggests that theory, in general, allows estimation of τ_c of macromolecules in supercooled water.

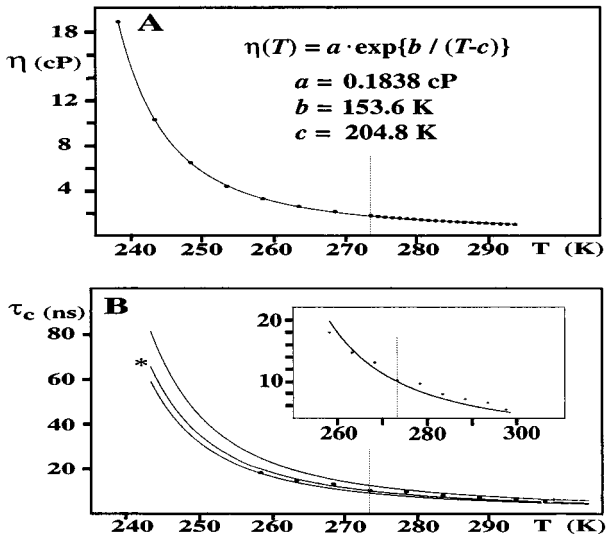

Figure 1. Overall rotational tumbling of globular proteins in supercooled water. The freezing point of water (273 K) is indicated. (A) Viscosity, η, of water as a function of *T*. The dots represent published values.[3] The fitted curve represents the indicated exponential function. (B) Rotational correlation time, τ_c, of ubiquitin[6] versus *T*. Experimental values[9] are represented by dots, and the middle curve (asterisk) was obtained from a fit of eq 1 yielding $r_H = 17.2$ Å. The upper ($r_H = 18.2$ Å) and the lower curve ($r_H = 16.6$ Å) enclose the experimental values shown at higher resolution in the insert. Fits were performed with SigmaPlot 4.0.

Here we present the first multidimensional NMR spectra acquired[9] for a protein (ubiquitin) in supercooled water. The good quality of ^{1}H NMR spectra (Figures 2a, S3, S4) shows that structure determinations of small proteins (<10 kDa) pursued below −10 °C will profit from homonuclear ^{1}H NMR. High-quality 2D [^{13}C,^{1}H]-HSQC (Figure 2b) at −15 °C and 3D HNCA at −11 °C (Figure 2c) show that heteronuclear resolved NMR[2d] serves well to obtain assignments. TROSY[10] is tailored for long τ_c; 2D [^{15}N,^{1}H]-TROSY (Figure 2d) shows that such spectroscopy is well suited below 0 °C (pronounced differential line broadening was observed[6] in ω_1,ω_2-$^1J_{NH}$-coupled HSQC at −15 °C, Figure 3B). For structure determinations in supercooled water, measurement of residual dipolar couplings[11] is attractive[12] since large τ_c may require deuteration.[13] Since bicelle systems are restricted to ambient *T*, we explored the Pf1 phage system.[14] 1% (0.5%) solutions in capillaries[6] can be cooled to −8 °C (−15 °C), i.e., at >0.5% phage the impact of capillaries[6] is reduced. Moreover,

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(1) Abbreviations used: NMR, nuclear magnetic resonance; 1D, 2D, 3D, one-, two-, three-dimensional; HNCA, NMR experiment correlating polypeptide backbone $^1H^N$, ^{15}N, and $^{13}C^\alpha$ chemical shifts; HSQC, heteronuclear single-quantum correlation; NOE, nuclear Overhauser effect; T_1, longitudinal nuclear spin relaxation time; $T_{1\rho}$, transverse nuclear spin relaxation time in the rotating frame; TROSY, transverse relaxation-optimized spectroscopy; dGTP, 2′-deoxyguanosine-5′-triphosphate; dTTP, 2′-deoxythymidine-5′-triphosphate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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(6) 0.8 mM solutions (50 mM K−PO$_4$, pH = 5.9) of ^{13}C/^{15}N labeled human ubiquitin comprising a C-terminal Ser-(His)$_6$-segment (Martek, MD) were put in glass capillaries (Wilmad, NJ, No. 1365-1.7) or 5 mm tubes (Wilmad, NJ, No. 528). $T = -7$ °C (5 mm tube) and −16 °C (capillary tube) could be reached without freezing. 3D HNCA was recorded with a sample in which the volume between the capillaries inside the 5 mm tube was also filled with protein solution; this allowed reaching −12 °C. Pf1 phage (ASLA, Riga, Latvia) solutions (0.5% and 1%; w/v) were prepared in capillaries (10 mM K−PO$_4$, pH = 7.0). Residual ^{15}N−^{1}H dipolar couplings were measured with a 1.5 mM solution of ^{15}N-labeled ubiquitin (Martek, MD) in a 5 mm tube containing 1.3% (w/v) phage at elevated ionic strength (10 mM K−PO$_4$, pH = 6.9, 250 mM NaCl); NMR lines broaden at lower ionic strength.[14] For detection of imino proton resonances, 10 mM aqueous solutions of dGTP and dTTP in capillaries were used (pH = 7.0).

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(9) NMR spectra were recorded on a VARIAN Inova750 spectrometer. *In capillaries:* ^{15}N T_1 and $T_{1\rho}$ with 1D schemes[8] extended for suppression of cross correlated relaxation[2d] (T_1-delays: 31, 95, 213, 290, 379, 480, 592, 852, 1000 ms; $T_{1\rho}$-delays: 8,16,23,31,47,55,62,78,94,125 ms; 7 kHz ^{15}N continuous wave spin-lock; total integrals of $^1H^N$ resonances between 8 and 9.5 ppm, which excludes side chain amides, were determined; T = 25,20,15,10,5,0,−5,−10,−15 °C.; total measurement time: 96 h). 2D [^{13}C,^{1}H]-HSQC ($t_{1,max}$ (^{13}C) = 22 ms, $t_{2,max}$ (^{1}H) = 71 ms, T = 25, 0, −8, −15 °C.; 30 h total). 2D [^{15}N,^{1}H]-TROSY ($t_{1,max}$ (^{15}N) = 49 ms, $t_{2,max}$ (^{1}H) = 48 ms, T = 25,15,5,−5,−6,−7,−8,−11,−15 °C.; 60 h total). ω_1,ω_2-$^1J_{HN}$-coupled HSQC ($t_{1,max}$ (^{15}N) = 49 ms, $t_{2,max}$ (^{1}H) = 48 ms, T = −15 °C.; 24 h). *In capillaries with void volume filled:*[6] 3D HNCA ($t_{1,max}$ (^{13}C) = 6 ms, $t_{2,max}$ (^{15}N) = 24 ms, $t_{3,max}$ (^{1}H) = 48 ms; T = 25, −11 °C.; 28 and 144 h, respectively). *Pf1 solution in 5 mm tube*: 2D [^{15}N,^{1}H]-HSQC without $^1J_{NH}$ decoupling along ω_1(^{15}N) ($t_{1,max}$ (^{15}N) = 46 ms, $t_{2,max}$ (^{1}H) = 48 ms, T = 25, −7 °C.; 6 and 24 h, respectively).

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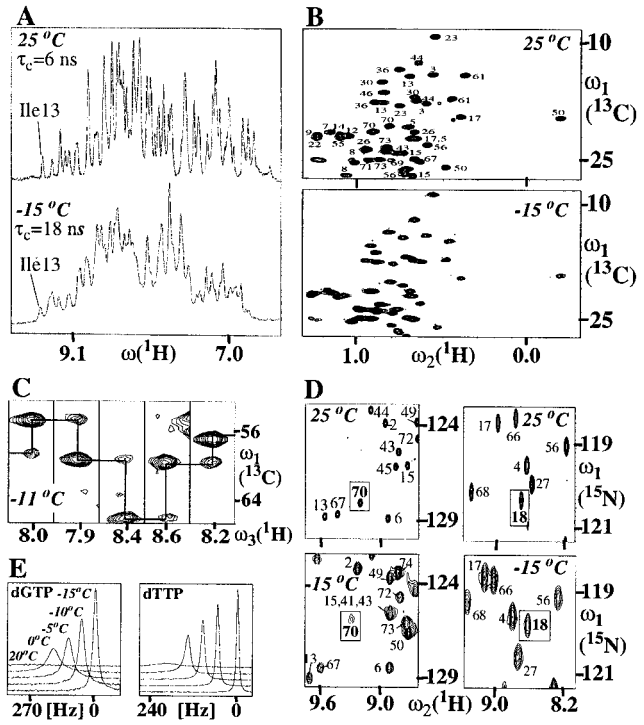

Figure 2. Multidimensional NMR in supercooled water. (A−D) for ubiquitin: (A) Downfield regions from 750 MHz jump−return 1D ^{1}H NMR spectra[2d] at 25 °C and −15 °C. τ_c is given (Figure 1). The $^1H^N$ resonance of Ile13 exhibits a width at half-height of 14 Hz at 25 °C and 32 Hz at −15 °C. With $^3J_{HN\alpha} = 9$ Hz, the natural line widths are about 5 and 23 Hz, respectively. (B) Regions from 2D [^{13}C,^{1}H]-HSQC[2a] spectra[9] containing the methyl signals. Assignments[16] are given at 25 °C. (C) [$\omega_1(^{13}C^\alpha)$,$\omega_3(^1H^N)$] strips of residues 28−32 from 3D HNCA[2d] recorded[9] at −11 °C. Sequential connectivities are indicated. (D) Regions from 2D [^{15}N,^{1}H]-TROSY[10] spectra[9] containing the signal of Val70 (boxed on the left) that broadens upon supercooling (lower left), and Glu18 exhibiting a large *positive* temperature coefficient below 0 °C for $^1H^N$ (boxed on the right). Shifts are in ppm and relative to DSS. (E) For dGTP (left) and dTTP (right): imino proton resonances from 1D jump-return ^{1}H NMR. The line widths (in Hz) are for dGTP at 20 °C: >200, 0 °C: 62, −5 °C: 37, −10 °C: 28, −15 °C: 18. Corresponding widths for dTTP: 90, 18, 12, 10, 8.

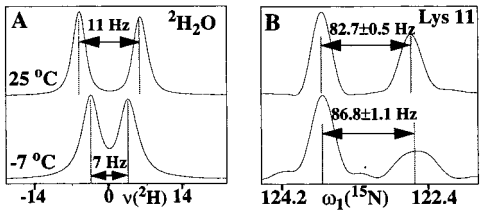

Figure 3. NMR in a supercooled dilute liquid crystalline medium with 1.3% Pf1 phage.[6,9,14] (A) The residual quadrupolar couplings of ^2H_2O are indicated. At low ionic strength the following couplings (in Hz) were obtained in capillary tubes.[6] 0.5% phage: 20 °C: 6, 0 °C:5, −15 °C:3. 1% phage: 20 °C:15, 0 °C:11, −8 °C:9. (B) ^{15}N−^{1}H couplings observed for Lys11 of ubiquitin[6,9] at 25 and at −7 °C. The coupling is reduced upon supercooling: $^1J_{NH} = -93.9$ Hz at 750 MHz[22] yields 11.2 and 7.1 Hz at 25 and −7 °C, respectively.

the residual quadrupolar coupling of ^2H_2O decreases[14] with T (Figure 3a). Nonetheless, we measured, for the first time, sizable residual dipolar couplings for structural refinement in a supercooled dilute liquid crystalline medium (Figure 3b).

Ubiquitin remains virtually unchanged between 25 °C and −15 °C, i.e., potential cold denaturation[16] is not observed. Nearly complete ^{15}N, $^1H^N$, and $^{13}C^\alpha$, and methyl assignments below −10 °C (Tables S2, S3) reveal that supercooling has little effect on these shifts,[15] and the coefficients of $^1H^N$ resonances do not indicate larger conformational rearrangements. $^1H^N$ of Glu18 is an outstanding exception. It exhibits a large *positive T* coefficient, $\Delta\delta/\Delta T$, of 9 ppb K^{-1} below −5 °C, while $\Delta\delta/\Delta T = -3$ ppb K^{-1} above 15 °C; it is the only $^1H^N$ shifted significantly upfield (0.06 ppm) at −15 °C (Figure 2d). The carbonyl oxygen of Glu18 is hydrogen bonded to $^1H^N$ of Asp21 in a type I turn,[17] and $\Delta\delta(^1H^N)/\Delta T$ of Asp21 decreases from −7 ppb K^{-1} at >15 °C to −1 ppb K^{-1} below −10 °C. Possibly, a local conformational change due to an increased population of the hydrogen bond occurs below −5 °C. The signal of Val70 shifts most upfield along $\omega_1(^{15}N)$ (1.6 ppm) and broadens upon supercooling (Figure 2d). Relaxation data[17] at 30 °C did not reveal a slow motion at Val70: it appears that the correlation time of a mode escaping detection at ambient T is shifted into the intermediate regime. Hence, NMR in supercooled water can provide novel insight by shifting correlation times into ranges that are well accessible by exchange spectroscopy[2b] or measurement of rotating frame relaxation times[8] or by freezing out conformations, e.g., the disulfide bond isomers of BPTI.[8,18]

Highest-quality NMR structures do not match the precision of highest-resolved (<1.0 Å) X-ray structures,[19] which allow checking on methodology for structural refinement, molecular dynamics simulations, and calculation of charge densities.[20] Thus, novel technology for highest-quality NMR structures is attractive. We address the unique potential of NMR in supercooled water for structural refinement of smaller proteins: reduced mobility may decrease NOE quenching, reduce conformational exchange broadening, and stall ring flipping[2b,c] enabling detection of distinct resonances for stereochemically equivalent ring protons. Reduced exchange of labile protons will allow extraction of additional structural constraints. Increased accuracy can also be expected for smaller nucleic acids including loops with non-base paired nucleotides: non-hydrogen bonded imino protons are readily observable at −15 °C (Figure 2e), thus serving to detect NOEs and/or residual dipolar ^{15}N−^{1}H couplings, and reduced exchange[21] of RNA 2′-hydroxyl protons will yield structural parameters for these protons in supercooled water.

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Supporting Information Available: I. Tables with ^{15}N relaxation parameters, ^{15}N, $^1H^N$, and $^{13}C^\alpha$ backbone, and ^{1}H and ^{13}C methyl chemical shifts. II. Details of sample preparation. III. ^{1}H NMR spectra, including 2D [^{1}H,^{1}H]-NOESY (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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