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.

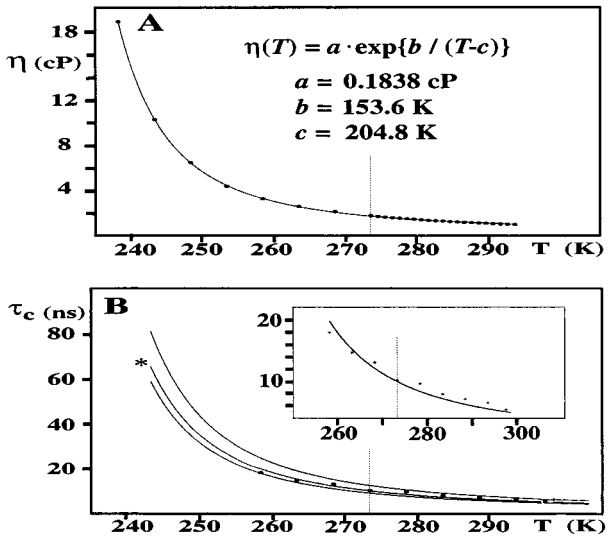

Figure 1. Overall rotational tumbling of globular proteins in supercooled water. The freezing point of water (273 K) is indicated. (A) Viscosity, η, of water as a function of *T*. The dots represent published values.[3] The fitted curve represents the indicated exponential function. (B) Rotational correlation 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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