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Protein dynamics in supercooled water: The search for slow motional modes

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Abstract

The impact of studying protein dynamics in supercooled water for identifying slow motional modes on the μs time scale is demonstrated. Backbone ^{15}N spin relaxation parameters were measured at −13 °C for ubiquitin, which plays a central role for signaling proteolysis, cellular trafficking and kinase activation in eukaryotic organisms. A hitherto undetected motional mode involving Val 70 was found, which may well play an important role for ubiquitin recognition. The measurement of rotating frame ^{15}N relaxation times as a function of the spin-lock field allowed determination of the correlation time of this motional mode, which would not have been feasible above 0 °C.

Abbreviations: NMR – nuclear magnetic resonance; r.f. – radiofrequency; CPMG – Carr-Purcell-Meiboom-Gill; NOE – nuclear Overhauser effect; T_1 – longitudinal spin-lattice relaxation time; T_2 – transverse spin-spin relaxation time; $T_{1\rho}$ – transverse spin relaxation time in the rotating frame; CW – continuous wave.

Many biological processes occur on the μs to ms time scale. This suggests that internal motions of proteins on that time scale may well be linked to function (Feher and Cavanagh, 1999; Ishima and Torchia, 2000; Volkman et al., 2001). NMR spectroscopy is uniquely suited to determine the correlation times, τ_{ex}, of such modes in solution. Particularly powerful are measurements of ^{15}N spin relaxation times in the rotating frame as a function of either (i) the CW r.f. spin-lock frequency ω_1 (Szyperski et al. 1993), (ii) the delay between the 180° r.f. pulses of a CPMG pulse train (Orekhov et al., 1995), or (iii) the ^{15}N r.f. carrier frequency in a constant relaxation time experiment (Akke and Palmer, 1996). We recently proposed NMR-based structural biology in supercooled water (Skalicky et al., 2000, 2001). Here we present the first spin relaxation study of a protein dissolved in supercooled water and show that this allows one to obtain novel insights into protein dynamics.

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The interpretation of a $T_{1\rho}^{-1}$ (ω_1) *versus* ω_1 profile does not depend on a particular spatial motional model. It suffices to consider the number of exchanging states, their populations and their chemical shifts. For a two-site exchange, $T_{1\rho}^{-1}$ (ω_1) is given by (Sandström, 1982; Szyperski et al., 1993):

$$\frac{1}{T_{1\rho}} = P_A P_B \Delta\Omega^2 \frac{1}{1 + (\omega_1 \tau_{ex})^2} + \frac{1}{T_{1\rho}^{D/CSA}} \quad (1)$$

where $\Delta\Omega$ is the shift difference between the two states A and B, P_i is the population of state i, ω_1 is the spin-lock frequency, τ_{ex} is the correlation time of the exchange process, $T_{1\rho}$ is the spin relaxation time in the rotating frame, and $T_{1\rho}^{D/CSA}$ is the r.f. independent $T_{1\rho}$. A fit of Equation 1 to experimental data yields τ_{ex}, $T_{1\rho}^{D/CSA}$ and $P_A P_B \Delta\Omega^2$. Determination of τ_{ex} requires (i) that ω_1 is varied around τ_{ex}^{-1}, and (ii) that the magnitude of $P_A P_B \Delta\Omega^2$ ensures a sufficiently large change of $T_{1\rho}(\omega_1)$ with varying ω_1. Current hardware affords up to ω_1 ~25 000 rad s^{-1}, i.e., the shortest measurable τ_{ex} is ~40 μs. Faster motions can hardly be (quanti-

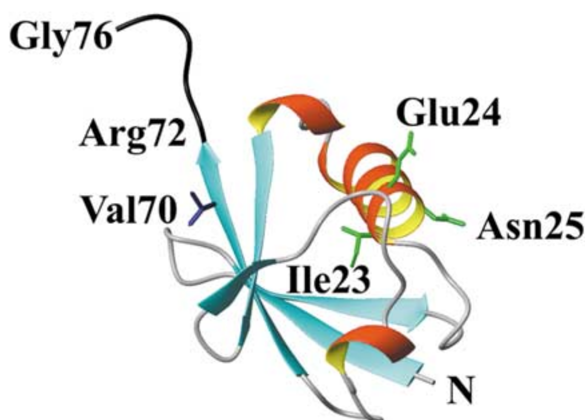

Figure 1. Ribbon drawing of ubiquitin derived from an NMR structure (Cornilescu et al., 1998; PDB 1D3Z). Residues for which slow conformational exchange was detected in supercooled water (−13 °C) are indicated. The motional mode at Val 70 (side chain depicted in blue) escaped detection at ambient temperature (Schneider et al., 1992; Tjandra et al., 1995; Fushman and Cowburn, 1998; de Alba et al., 1999; Carlomango et al., 2000; Meiler et al., 2001; Tolman, et al., 2001). The C-terminal segment 72-76 (the 'active site'; e.g., Pickart, 2001) is flexibly disordered in solution and depicted in black.

tatively) assessed, and the same holds if $\Delta\Omega$ is too small or if the populations are too strongly skewed. If laboratory frame relaxation is not measurably affected, such 'μs-modes' may well escape detection at ambient temperature.

Upon supercooling, the free energies of the exchanging states, and thus the populations, may change. A shift in populations is manifested primarily in a change of the observed averaged chemical shift in the fast exchange limit (Sandström, 1982). In particular, if the transition from major to minor conformer is *exothermic*, supercooling favors the formation of the minor conformer (itself being entropically disfavored). In turn, this leads to an increase of the product $P_A P_B$ in Equation 1, and thus to increased excess line broadening. Furthermore, exchange rate constants are reduced at lower temperature, which leads to increased line broadening at fast exchange (Sandström, 1982). Supercooling may thus shift both populations and τ_{ex} into ranges that enable accurate characterization by NMR.

The 76-residue protein ubiquitin (Figure 1) is found in all eukaryotic cells. Ubiquitin is related to signaling proteolysis, cellular trafficking and kinase activation (Pickart, 2001), and its amino acid sequence is extremely conserved (Haas and Siepmann, 1997). Despite its central biological role, ubiquitin's molecular mode(s) of action has only been partly elucidated (Pickart, 2001). We measured ^{15}N relaxation parameters for human ubiquitin at 25 °C and −13 °C in order to search for hitherto uncharacterized slower motional modes. Using established criteria (e.g., de Alba et al., 1999), three residues were identified with excess ^{15}N line broadening. The resonance of Val 70 is broadened solely in supercooled water (Figure 2) but not at 25 °C, unraveling a motion that has not been detected in any of the previous comprehensive studies (Schneider et al., 1992; Tjandra et al., 1995; Fushman and Cowburn, 1998; de Alba et al., 1999; Carlomagno et al., 2000; Meiler et al., 2001; Tolman, et al., 2001). Moreover, supercooling allowed us to determine that $\tau_{ex} = 133 \pm 28$ μs at −13 °C (Figure 3). At both temperatures and in agreement with previous studies (Schneider et al., 1992; Tjandra et al., 1995; Fushman and Cowburn, 1998; de Alba et al., 1999; Carlomagno et al., 2000; Meiler et al., 2001; Tolman et al., 2001) slow exchange is detected for Ile 23 and Asn 25. Excess broadening is small for Ile 23 and Asn 25 at 25 °C (∼1–2 Hz) (de Alba et al., 1999). At −13 °C, however, the excess broadening is much larger (Figure 2), so that the bound $\tau_{ex} < 400$ μs could be derived for the two residues. The signal of Glu 24 is broadened beyond detection at both temperatures. Importantly, the quantitative assessment of τ_{ex} of the motional modes affecting Ile 23, Asn 25, and Val 70 would not have been feasible at temperatures above the freezing point of water. ^{15}N spin relaxation times measured at 1 °C reveal that excess broadening is small for all those residues (Figure 2B). The onset of excess broadening is registered for Val 70 but the broadening remains too small to ensure accurate determination of τ_{ex}.

The C-terminal heptapeptide segment of ubiquitin comprising residues Val 70 to Gly 76 is crucial for interaction (Pickart, 2001) with the ubiquitin activating enzyme (Haas et al., 1997), ubiquitin conjugating enzymes (Miura et al., 1999) and deubiquinating enzymes (Sakamoto et al., 1999; Wilkinson et al., 1999). In particular, Val 70 participates in the formation of a hydrophobic patch that confers specificity (Wilkinson et al., 1999). It may thus well be that we have identified and characterized a motional mode close to the 'active site' that is of functional importance. In contrast, we found no indication in current literature that the motional mode(s) at residues 23–25 are of functional importance.

Very recently, a high-pressure NMR study (Kitahara et al., 2001) implicated a second folded conformer of ubiquitin (15% abundance at 25 °C and 1 atm.) of potential functional importance. This mi-

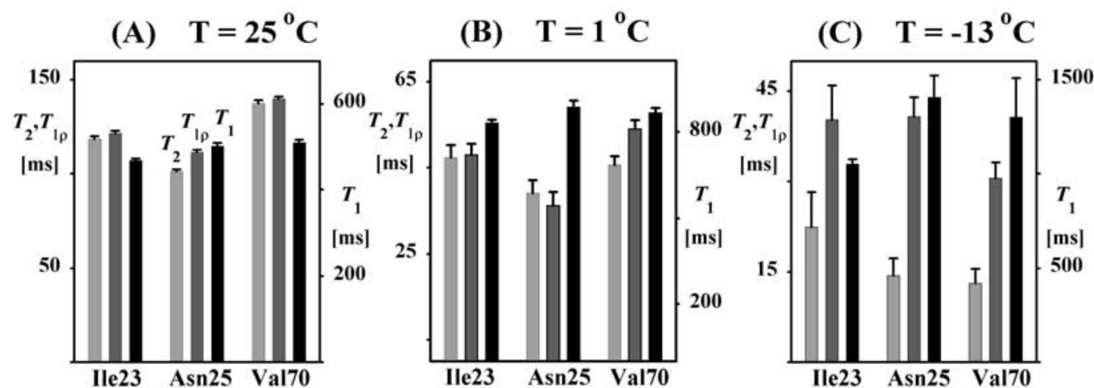

Figure 2. T_2 ( ), $T_{1\rho}$ ( ) (ω_1 = 27 600 rad s^{-1}) and T_1 (■) ^{15}N spin relaxation times of Ile 23, Asn 25 and Val 70 at 25 °C (A), 1 °C (B), and at −13 °C (C). Conformational exchange broadening can be inferred if $T_2 < T_{1\rho}$. Vertical bars represent the experimental errors. Steady-state ^{15}N{^{1}H} NOEs for these residues (25 °C: Ile 23, 0.86; Asn 25, 0.84; Val 70, 0.89. −13 °C: Ile 23, 0.78; Asn 25, 0.88; Val 70, 0.94) indicate that fast ps to ns motions are spatially quite restricted at both temperatures. At 25 °C, NMR data were acquired in a Shigemi tube using a 2 mM solution (50 mM K-PO$_4$, pH = 5.9) of ^{2}H/^{13}C/^{15}N labeled human ubiquitin (Martek, MD). For 1 °C and −13 °C, this solution was centrifuged (14 000 × G; 30 min) and put in eight glass capillaries (Skalicky et al., 2001) with an outer diameter of 1.0 mm (Wilmad, NJ, Cat. No. 1365–1.0). NMR spectra were acquired on a VARIAN INOVA 600 spectrometer. T_1, T_2 and $T_{1\rho}$ measurements were recorded with pulse schemes (Szyperski et al., 1993) extended for suppression of cross-correlated relaxation. ^{15}N{^{1}H} NOEs were acquired as described (Farrow et al., 1994). At 25 °C: T_1-delays: 25, 186, 346, 507, 668, 828, 989, 1149 ms; T_2-delays: 8, 16, 32, 48, 64, 72, 88, 105 ms; $T_{1\rho}$-delays: 8, 24, 36, 52, 64, 80, 92, 108 ms at ω_1 = 27 600 rad s^{-1}. Total measurement time: 60 h. At 1 °C: T_1-delays: 25, 186, 346, 507, 668, 828, 989, 1150, 1305 ms; T_2-delays: 8, 16, 24, 32, 40, 48, 64, 72, 80 ms; $T_{1\rho}$-delays: 8, 20, 36, 48, 60, 72, 88, 100, 112 ms at ω_1 = 27 600 rad s^{-1}. Total measurement time: 42 h. At −13 °C: T_1-delays: 25, 206, 386, 567, 748, 929, 1109, 1290 ms; T_2-delays: 8, 16, 24, 40, 48, 56, 64, 80 ms; For $T_{1\rho}(\omega_1)$ measurements of Val 70 (δ(^{15}N) = 126.6 ppm), and for Ile 23 (δ(^{15}N) = 122.7 ppm) and Asn 25 (δ(^{15}N) = 123.3 ppm), the r.f. carrier was set to 126.6 ppm and 123.0 ppm, respectively. ω_1-values: 3.1, 4.2, 5.1, 6.0, 7.5, 14.3, 27.6 [10^3 rad s^{-1}]. $T_{1\rho}$-delays: 0, 4, 8, 12, 16, 20, 24 ms. Total measurement time: 244 hours. For studies in which the ^{15}N chemical shifts are significantly off-resonance relative to the carrier position, the application of an off-resonance correction is recommended (Davis et al., 1995).

nor conformer exhibits an unusually large shift of the ^{15}N chemical shift of Val 70 (resonating 11.2 ppm upfield of the major conformer; Kitahara et al., 2001). Furthermore, it is in fast exchange with the major conformer and is supposed to possess a more extended flexible disordered C-terminus starting at Val 70 (see Figure 1). Indeed, the local backbone r.m.s.d values of residues 70 and 71 are increased about two-fold in an NMR structure (Cornilescu et al., 1998; PDB 1D3Z) when compared to all other residues 2 to 69. This is expected if conformational exchange leads to partial ^{1}H-^{1}H NOE quenching at these two residues. For the same resonance of Val 70 a 1.6 ppm upfield shift is observed when cooling from 25 °C to −15 °C. Hence, it is tempting to speculate that we are monitoring the interconversion of these two postulated conformers of ubiquitin in supercooled water. In agreement with a two-state scenario (Sandström, 1982; Szyperski et al., 1993), the $T_{1\rho}^{-1}$ (ω_1) profile (Figure 3) is (within error) of Lorentzian shape. However, in contrast to the high-pressure study (Kitahara et al., 2001) the ^{15}N{^{1}H} NOE of Val 70 is not decreased at −13 °C (Figure 2). The 1.6 ppm upfield shift would imply ~30% (15% + 1.6/11.2 × 100%) minor form at −13 °C. Since the ^{15}N{^{1}H} NOE for Val 70 in the minor conformer is ~0.5 (Kitahara et al., 2001), a measurable decrease could be expected at −13 °C. Moreover, the value measured for $P_A P_B \Delta\Omega^2$ (Figure 3) is much smaller than expected: with $\Delta\Omega$ = 4280 rad s^{-1} (11.2 ppm), P_A = 0.7 and P_B = 0.3, one obtains $P_A P_B \Delta\Omega^2 = 3.7 \times 10^6$ rad^2 s^{-2} which is much larger than 3.9×10^5 rad^2 s^{-2} as obtained at −13 °C (Figure 3). Finally, the chemical shift changes observed at high pressure for other residues (Kitahara et al., 2001) do not correlate well with those registered upon supercooling (Skalicky et al., 2000). We thus conclude the dynamic phenomena detected at very high pressure and in supercooled water are not identical.

In general, this study raises the question of how frequently motional modes escape detection in routinely performed ^{15}N spin relaxation studies at ambient temperature. Apart from possibly missing functionally important motions, this is of interest for recruiting spin relaxation parameters to determine thermodynamic quantities (Akke et al., 1993; Yang et al.,

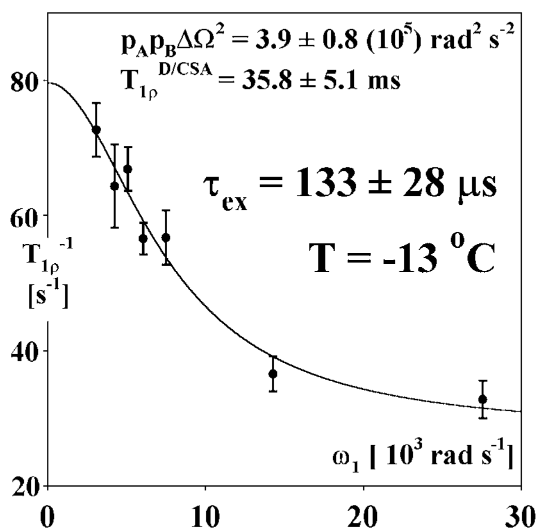

Figure 3. $T_{1\rho}^{-1}$ of ^{15}N of Val 70 *versus* the spin-lock frequency, ω_1, determined at T = −13 °C. The solid curve represents the fit of Equation 1 to the experimental data (●). Vertical bars represent the experimental errors.

1997; Wand, 2001). Both our study and the dynamic interpretation of residual dipolar couplings (Meiler et al., 2001) indicate that an important fraction of internal modes may escape detection in routine ^{15}N relaxation studies. Indeed, the residual dipolar coupling measured for the N-H moiety of Val 70 (Ottiger and Bax, 1998) revealed a so far unexplained deviation from the value predicted from the X-ray crystal structure, and it seems likely that this is due to the motional mode characterized here in supercooled water. Interestingly, a deviation from the predicted value has also been detected for the anisotropy of the carbonyl chemical shift of Val 70 (Cornilescu and Bax, 2000). Furthermore, the determination of activation parameters for a given τ_{ex} as described by Blackledge et al. (1993) could likewise be pursued in supercooled water and would then allow one to calculate the corresponding correlation time at ambient temperature. Overall, we conclude that studies in supercooled water can enhance our understanding of protein dynamics and its role for function (Akke et al., 1998; Feher and Cavanagh, 1999; Ishima and Torchia, 2000; Volkman et al., 2001).

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