Aromatic Ring-Flipping in Supercooled Water: Implications for NMR-Based Structural Biology of Proteins

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Abstract: We have characterized, for the first time, motional modes of a protein dissolved in supercooled water: the flipping kinetics of phenylalanyl and tyrosyl rings of the 6 kDa protein BPTI have been investigated by NMR at temperatures between −3 and −16.5 °C. At \( T = -15 \) °C, the ring-flipping rate constants of Tyr 23, Tyr 35, and Phe 45 are smaller than 2 s\(^{-1}\), i.e., flip-broadening of aromatic NMR lines is reduced beyond detection and averaging of NOEs through ring-flipping is abolished. This allows neat detection of distinct NOE sets for the individual aromatic \(^1\)H spins. In contrast, the rings of Phe 4, Tyr 10, Tyr 21, Phe 22, and Phe 33 are flipping rapidly on the chemical shift time scale with rate constants being in the range from approximately \( 10^2 \) to \( 10^5 \) s\(^{-1}\) even at \( T = -15 \) °C. Line width measurements in 2D \([\text{H},\text{H}]-\text{NOESY}\) showed that flipping of the Phe 4 and Phe 33 rings is, however, slowed to an extent that the onset of associated line broadening in the fast exchange limit is registered. The reduced ring-flipping rate constant of Phe 45 in supercooled water allowed very precise determination of Eyring activation enthalpy and entropy from cross relaxation suppressed 2D \([\text{H},\text{H}]-\text{exchange spectroscopy}. This yielded \( \Delta H^\ddagger = 14 \pm 0.5 \text{ kcal-mol}^{-1} \) and \( \Delta S^\ddagger = -4 \pm 1 \text{ cal-mol}^{-1}\cdot\text{K}^{-1} \), i.e., values close to those previously derived by Wagner and Wüthrich for the temperature range from 4 to 72 °C (\( \Delta H^\ddagger = 16 \pm 1 \text{ kcal-mol}^{-1} \) and \( \Delta S^\ddagger = 6 \pm 2 \text{ cal-mol}^{-1}\cdot\text{K}^{-1} \)). The preservation of the so far uniquely low value for \( \Delta S^\ddagger \) indicates that the distribution of internal motional modes associated with the ring flip of Phe 45 is hardly affected by lowering \( T \) well below 0 °C. Hence, if a globular protein does not cold denature, aromatic flipping rates, and thus likely also the rates of other conformational and/or chemical exchange processes occurring in supercooled water, can be expected to be well estimated from activation parameters obtained at ambient \( T \). This is of keen interest to predict the impact of supercooling for future studies of biological macromolecules, and shows that our approach enables one to conduct NMR-based structural biology at below 0 °C in an unperturbed aqueous environment. A search of the BioMagResBank indicated that the overwhelming majority of the Phe and Tyr rings (>95%) are flipping rapidly on the chemical shift time scale at ambient \( T \), while our data for BPTI and activation parameters available for ring-flipping in Iso-2-cytochrome c reveal that in these smaller proteins a total of six out of seventeen rings (~35%) are “frozen in” at \( T = -15 \) °C. This suggests that a large fraction of Tyr and Phe rings in globular proteins that are flipping rapidly on the chemical shift time scale at ambient \( T \) can be effectively slowed in supercooled water. The present investigation demonstrates that supercooling of protein solutions appears to be an effective means to (i) harvest potential benefits of stalled ring-flipping for refining NMR solution structures, (ii) recruit additional aromatic rings for investigating protein dynamics, and (iii) use multiple slowly flipping rings to probe cold denaturation. The implications for NMR-based structural biology in supercooled water are addressed.

Introduction

We have very recently demonstrated the feasibility of NMR\(^1\)-based structural biology in supercooled water\(^2\) and addressed its unique potential for (i) structural refinement of smaller proteins and nucleic acids and (ii) obtaining novel insights into biomolecular dynamics, hydration, and cold denaturation. Most importantly, the use of supercooled water\(^3\) allows reaching temperatures below 0 °C in an unperturbed aqueous environment, i.e., without adding chemicals or applying very high pressure for lowering the freezing point of water. At sub-zero temperatures, reduced internal mobility can yield reduced NOE quenching, reduced chemical exchange of labile protons, and reduced conformational exchange including the flipping\(^4\) of aromatic rings. Since aromatic side chains quite generally constitute a sizable fraction of a protein’s hydrophobic core, the reduction of aromatic ring-flipping rates in supercooled water is of particular interest in view of NMR structures with the highest possible accuracy. Furthermore, aromatic ring-flipping in the molecular core is intimately connected to larger amplitude motional modes. Assessing the ring-flipping rate constants in supercooled water can thus be expected to yield valuable new

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\(^{1}\) Abbreviations used: NMR, nuclear magnetic resonance; 1D, 2D, one- and two-dimensional; NOE, nuclear Overhauser effect; NOESY, Nuclear Overhauser Enhancement Spectroscopy; o.d., outer diameter; r.f., radio frequency; BMRB, BioMagResBank; BPTI, bovine pancreatic trypsin inhibitor; EXSY, exchange spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulphonate.


