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 tyrosinyl 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 [^{1}H,^{1}H]-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 [^{1}H,^{1}H]-exchange spectroscopy. This yielded $\Delta H^{\ddagger} = 14 \pm 0.5$ kcal·mol^{-1} and $\Delta S^{\ddagger} = -4 \pm 1$ cal·mol^{-1}·K^{-1}, i.e., values close to those previously derived by Wagner and Wüthrich for the temperature range from 4 to 72 °C ($\Delta H^{\ddagger} = 16 \pm 1$ kcal·mol^{-1} and $\Delta S^{\ddagger} = 6 \pm 2$ cal·mol^{-1}·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,[5] 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-sulfonate.

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insights into protein dynamics and folding. In this paper we experimentally investigate, for the first time, the flipping of aromatic rings of a protein dissolved in supercooled water.

Due to anisotropic shielding stereochemically equivalent aromatic Tyr and Phe ring protons (e.g., the δ^1- and δ^2-protons) often exhibit different chemical shifts if the ring is placed in a fixed orientation in the interior of a protein. At ambient temperatures *T*, however, rapid ring-flipping due to rotation about the $C^\beta-C^\gamma$ bond (χ^2-angle[6]) usually averages these chemical shifts. This has two major consequences. First, flipping rate constants can be assessed for these fast flipping rings only for a smaller subset exhibiting flip-broadened resonances. Since the chemical shift differences associated with the flip are quite generally not known, the registered line broadening can, however, usually not be directly translated into flipping rates, i.e., only ranges of rate constants can be inferred. In effect, the predominance of rapid ring-flipping at ambient *T* thus prevents us from using the majority of a proteins' aromatic rings as probes to study dynamics. Second, NOE-derived distance constraints for structure determinations which involve ring protons with degenerate chemical shifts need to be (i) referred to a pseudoatom centrally located between the ring protons, and a large pseudoatom correction of up to 2.4 Å is added to the constraints,[5,7a] or (ii) treated as ambiguous constraints.[7b] In fact, the magnitude of this correction is comparable to those required for NOE constraints derived for Val and Leu methyl groups with either degenerate ^{1}H chemical shifts or absent stereospecific assignments.[5,7a] Such very large pseudoatom corrections have long been recognized as a caveat for generating high-quality NMR structures, and a suite of different approaches has thus been developed to stereospecifically assign these methyls.[7,8] In contrast to the isopropyl methyls of Val and Leu, symmetry-related aromatic protons are obviously not prochiral. Hence, their chemical shifts necessarily become equivalent through fast rotation about χ^2, and only suitable reduction of the ring-flipping rate can yield the chemical shift dispersion required to avoid pseudoatom corrections or the use of ambiguous distance constraints.[7b] In addition, aromatic ring-flipping introduces conformational exchange mediated magnetization transfer into NOESY,[9,10] the major source of information for NMR structure determinations. For biological macromolecules, cross-peaks from conformational exchange and dipolar cross relaxation exhibit the same sign.[10] Hence, NOESY cross-peaks involving conformational exchange during the mixing period are a priori indistinguishable from those arising from pure cross relaxation. If the ring-flipping is slow on the chemical shift time scale so that distinct resonances are observed, but fast enough to transfer longitudinal magnetization during the NOE mixing time, it can lead to direct exchange peaks and, due to the joint action of exchange and ^{1}H$-^1$H dipolar interaction, to averaging of cross-peak intensities registered for the pairs of exchanging spins. By a cascade of magnetization transfers involving a NOE-relay, a ring-flip, and a second NOE-relay, ring-flipping may also generate conformational exchange mediated[9,10] cross-peaks for (nonaromatic) proton pairs that are located in the vicinity of the flipping ring. Hence, if ring-flipping is not considered for data interpretation, NOE cross-peaks affected by the exchange may well translate into erroneously short distance constraints, potentially yielding apparently well-refined but incorrect NMR structures.[11] Importantly, the distance between the two δ- or ϵ-protons in Phe or Tyr is quite long (4.3 Å). Hence, even at short mixing times ring-flipping may generate cross-peaks between protons separated by 7 Å or more, which is well above the quite generally observed NOE detection limit of 5−6 Å. These potential complications of ring-flipping for obtaining high-quality NMR solution structures have been experimentally documented by Macura, Markley, and co-workers,[11] and they foreseeably become of key importance for future attempts to derive new generation NMR structures with further improved accuracy.

The flipping of Phe and Tyr rings[4,11−14] has been extensively explored for the bovine pancreatic trypsin inhibitor (BPTI) by Wagner, Wüthrich, and co-workers[12] in the 1970s and 1980s. These studies[12] have largely shaped our view of this important dynamic phenomenon in globular proteins, and BPTI, which comprises four phenylalanyl and four tyrosinyl residues, still represents the protein with the most comprehensive characterization of ring-flipping over a large *T* range from 4 to 72 °C. In view of the completeness of this body of experimental data, we decided to investigate ring-flipping in BPTI dissolved in supercooled water; experimental data were acquired down to a temperature of $T = -16.5$ °C. The implications of our findings for the NMR-based structural biology of proteins in supercooled water are addressed.

Materials and Methods

Sample Preparation. NMR measurements were performed with 6 mM solutions of BPTI (Sigma-Aldrich, MO) at pH 3.5. This pH was previously chosen for the high-quality NMR solution structure determination of BPTI.[15] Four NMR samples were prepared: "sample I", with 90% H_2O/10% D_2O as the solvent and a 5 mm NMR tube; "sample II", with D_2O as the solvent and a 5 mm NMR tube; "sample c1.7", with D_2O as the solvent and three 1.7 mm o.d. glass capillary tubes;[2] and "sample c1.0", with D_2O as the solvent and ten 1.0 mm o.d. glass capillary tubes. The capillaries were placed within a regular 5 mm NMR tube.[2] The volume of the protein solution within the region assessed by the probe's detection coil was respectively 0.41 and 0.28 for samples c1.0 and c1.7 when compared with a regular sample. For additional information see the Supporting Information.

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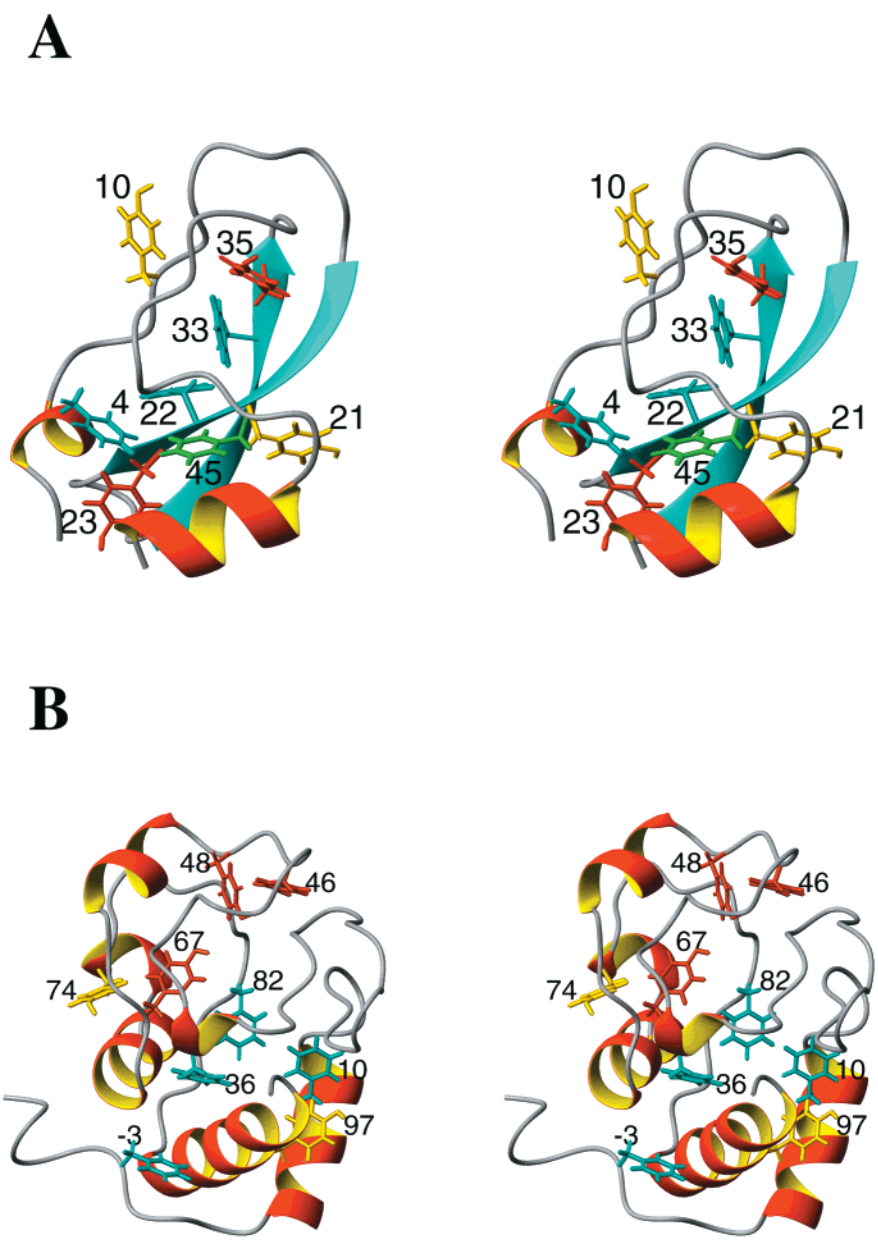

Figure 1. Stereo presentations derived form X-ray crystal structures of phenylalanyl and tyrosinyl rings in (A) BPTI [28a] and (B) Iso-2-cytochrome *c*.[28b] The polypeptide backbones are shown as ribbon drawings. Tyr and Phe rings, for which activation parameters for the ring flip have been published,[12,14b] are depicted in red and green, respectively. Tyr and Phe rings, which are either rapidly flipping on the chemical shift time scale at ambient *T* or for which no activation parameters were measured, are displayed in yellow and cyan, respectively. The numbers of the residues comprising the aromatic rings are indicated.

NMR Spectroscopy. NMR experiments were recorded on Varian Inova 750 or Inova 600 spectrometers, processed with the program PROSA,[16] and analyzed with the program XEASY.[17] Sample temperatures in the spectrometers were calibrated with use of the ^{1}H resonances of methanol, and supercooling of NMR samples was monitored as described.[2]

Eight 2D [^{1}H,^{1}H]-NOESY spectra[10] were recorded with a mixing time, τ_{mix}, of 40 ms at a 750 MHz ^{1}H spectrometer frequency and at *T* (sample) = −15 °C (c1.0), −13 °C (c1.7), −11 °C (c1.7), − 9 °C (c1.7), −7 °C (c1.7), −5 °C (c1.7), −3 °C (c1.7), and 36 °C (c1.7). To allow for a direct comparison of relative cross-peak intensities at *T* = −15 and 36 °C, two NOESY spectra were recorded in 18 and 36 h, respectively, at 600 MHz for which τ_{mix} was scaled with $1/\tau_c$, where τ_c represents the correlation time for the overall rotational tumbling (Figure 2). τ_c(−15 °C) ∼ 12.5 ns and τ_c(36 °C) ∼ 3 ns lead us to set τ_{mix}(−15 °C) = 15 ms and τ_{mix}(36 °C) = 70 ms, respectively. The total NOESY measurement time was 241 h. Three 2D [^{1}H,^{1}H]-TOCSY spectra[10] (sample c1.0, spin-lock r.f. field strength = 10 kHz) were recorded at 600 MHz and at *T* (sample) = −14.5 °C (c1.0) with τ_{mix} = 38 ms and at *T* (c1.0) = −7.5 °C with τ_{mix} = 38 or 55 ms. The total TOCSY measurement time was 24 h. Eight series of three or four cross relaxation suppressed 2D [^{1}H,^{1}H]-EXSY spectra[18] were recorded, if not indicated otherwise, at 600 MHz and at *T* (sample) = −16.5 °C (c1.0), −14.5 °C (c1.0), −12.5 °C (c1.0), −9.5 °C (c1.7, 750 MHz), −9.5 °C (c1.0), −7.5 °C (c1.0), −5.5 °C (c1.0), and −3.5 °C (c1.0). The total EXSY measurement time was 192 h. 2D [^{13}C,^{1}H]-HSQC spectra[10] were recorded in 48 h at 750 MHz at *T* (sample) = −6 °C (II) and 25 °C (II).

Data Analysis. Linear regression analyses were performed with the program SigmaPlot 4.0. The flipping rate, k_{flip}, of Phe 45 of BPTI was

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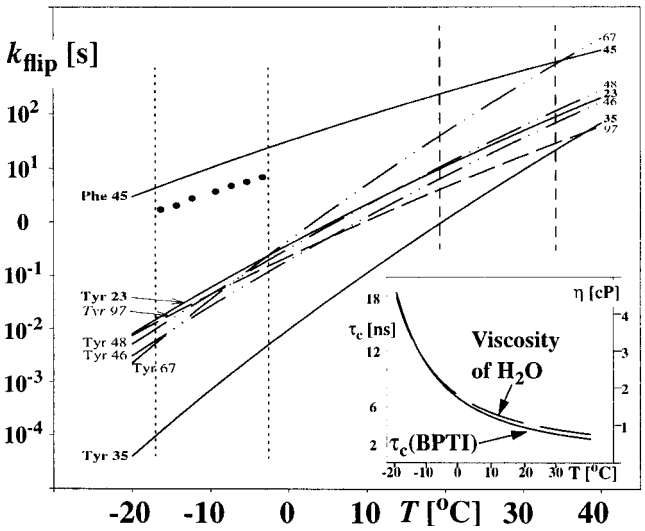

Figure 2. Semilogarithmic plot of ring-flipping rate constants, k_{flip}, calculated from published activation parameters[12,14] versus temperature, T, for Tyr 23, Tyr 35, and Phe 45 (indicated in bold) of BPTI (solid lines), for Tyr 46, Tyr 48, and Tyr 67 of Iso-2-cytochrome *c* (dashed−dotted lines), and for Tyr 97 (indicated in italics) of horse Cytochrome *c* (dashed line). Dashed vertical lines are placed at $T = 20$ and 35 °C to indicate the T-range commonly used for NMR structure determinations, and dotted vertical lines are drawn at $T = -3$ and −17 °C to indicate the T-range assessed here in supercooled water. For Phe 45 of BPTI (uppermost curve), the rate constants experimentally determined for the present study are shown as filled circles. The difference between prediction and measurement reflects a slightly smaller value for $\Delta S^{\ddagger}$ (see also Figure 6). The following values of $\Delta H^{\ddagger}$ [kcal·mol^{-1}] and $\Delta S^{\ddagger}$ [cal·mol^{-1}·K^{-1}] from literature[12,14] were used for calculating k_{flip}. BPTI: Tyr 23 (26, 35), Tyr 35 (37, 68), Phe 45 (16, 6). Iso-2-cytochrome *c*: Tyr 46 (28, 41), Tyr 48 (28, 42), Tyr 67 (36, 72). Cytochrome *c*: Tyr 97 (23, 23). The insert displays, for the same T-range from −20 to 40 °C, the viscosity, η, of supercooled water[2,3] (scale shown on the right) as well as the correlation time of the overall rotational tumbling, τ_c, of BPTI (scale shown on the left) calculated from hydrodynamic theory.[2,20a] The two curves were scaled so that the dominant influence of η on the T-dependence of τ_c (eq 4) is made apparent. Flipping rate constants were calculated from activation parameters using $1/k_{flip} = h/(k_B T) \exp[(\Delta H^{\ddagger}/RT - \Delta S^{\ddagger}/R)]$.

determined from both the 2D [^{1}H,^{1}H]-EXSY[18] and the 2D [^{1}H,^{1}H]-NOESY spectra.[10] To extract k_{flip} from EXSY with varying mixing times, the relation

$$\ln[-2V_C/(V_C + V_D) + 1] = -2k_{flip}\tau_{mix} \quad (1)$$

was fitted by linear regression to the experimental data, where V_D and V_C represent the diagonal and cross-peak volumes, respectively. From NOESY, k_{flip} was determined for a given mixing time by using the expression

$$k_{flip} = [-1/(2\tau_{mix})] \ln[(\kappa - 1)/(\kappa + 1)] \quad (2)$$

where $\kappa = V_D/V_C$. Eyring plots were obtained by fitting the Eyring equation[19] to the experimental data, i.e.,

$$\Delta G^{\ddagger}/T = R \ln[k_B T/(hk_{flip})] \quad (3)$$

versus $1/T$, where R, k, and h are the universal gas constant, Boltzmann's constant, and Planck's constant, respectively. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were assumed to be independent of T.

Correlation Time for the Overall Rotational Tumbling in Supercooled Water. We have recently demonstrated[2] that hydrodynamic theory[20] allows accurate prediction of the correlation time of the overall rotational tumbling of globular proteins dissolved in supercooled water. In addition, previous ^{13}C and ^{15}N nuclear spin relaxation measurements[21] at ambient temperature showed that BPTI may be well approximated as a spherical molecule in the framework of hydrodynamic theory. We thus calculated the correlation time for the overall rotational tumbling of BPTI using the well-established relation[20a]

$$\tau_c = 4\pi[\eta(T)]r_H^3/3k_B T \quad (4)$$

r_H is the effective radius with $r_H = [3\bar{V}M/(4\pi N_A)]^{1/3} + r_w$, 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.[20a]

Results and Discussion

The NMR observation of aromatic ring-flipping has been amply documented,[11−14] manifesting its general importance. To the best of our knowledge, however, the Eyring activation[19] parameters have so far been determined for only seven aromatic rings occurring in three different proteins, i.e., for Tyr 23, Tyr 35, and Phe 45 of BPTI[12] (comprising a total of four Tyr and four Phe), for Tyr 97 of Cytochrome *c*[14a] (in total four Tyr and four Phe), and for Tyr 48, Tyr 46, and Tyr 67 of Iso-2-cytochrome *c*[14b] (in total four Phe and five Tyr) (Figure 1). Although the appropriateness of Eyring's theory[19] for describing reactions of biological macromolecules can be called in question,[12g] the determination of the Eyring activation parameters represents an efficient way to parametrize the T-dependence of experimentally determined rate constants. Here we adopt this operational view and thus refrain from interpreting $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values in terms of molecular pictures at atomic resolution.

Prediction of Ring-Flipping Rates in Supercooled Water. We used the activation parameters of BPTI, Cytochrome *c*, and Iso-2-cytochrome *c* to predict the corresponding ring-flipping rates at temperatures below 0 °C (Figure 2). This indicated that all above listed tyrosine rings flip with rate constants, k_{flip}, smaller than 0.1 s^{-1} at temperatures below −7 °C, i.e., less than 1% of these rings are expected to flip within 100 ms at $T < -7$ °C. This implies virtual stalling of Tyr flipping on (i) the chemical shift time scale, which enables observation of aromatic NMR lines being unaffected by the flipping, and (ii) the NOE mixing time scale (<100 ms) commonly employed for structure determinations, which abolishes generation of conformational exchange affected or mediated[9,10] peaks. As an apparent exception, the ring of Phe 45 of BPTI is predicted to exhibit flipping rate constants in the range 26 s^{-1} > k_{flip} > 5 s^{-1} for −2 °C < T < −15 °C, offering itself to be studied by exchange spectroscopy (EXSY[10,18]).

^{1}H NMR of BPTI in Supercooled Water. Four different 6 mM NMR samples of BPTI were prepared for the present study (see Methods). The lowest temperatures could be reached with sample c1.0 (Figure 3). At $T = -15.5$ °C, the solutions in all 10 capillaries remained liquid, and at $T = -16.5$ and −18 °C respectively five and one capillary contained solution in a liquid state. $T = -13$ and −6.5 °C could be reached with sample c1.7, and samples I and II, respectively. Considering that the melting point of D_2O is $T = 3.82$ °C (at a pressure of 101.325 kPa),[22]

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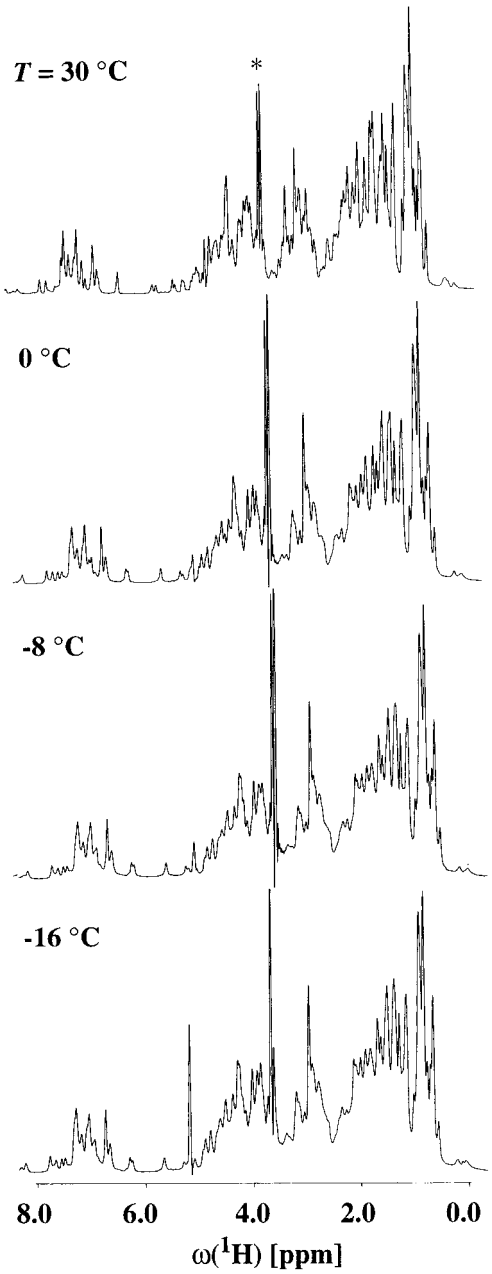

Figure 3. 1D ^{1}H NMR spectra recorded for BPTI at temperatures between 30 (top) and −16 °C (bottom). A signal arising from a lower molecular weight impurity is marked with an asterisk. ^{1}H chemical shifts are relative to DSS.

the use of the 1.0 mm capillaries thus allowed supercooling of the protein solution by about 20 °C. 1D ^{1}H NMR shows that BPTI is not cold denaturing even at $T = -16$ °C (Figure 3), and high-quality 2D NOESY and TOCSY spectra yielded complete assignments of the aromatic ^{1}H resonances down to $T = -15$ °C (Table S1, Figure 4). The near identity of (i) the aromatic ^{1}H chemical shifts (Table S1), (ii) the methyl ^{1}H and ^{13}C chemical shifts (Table S2), and (iii) the backbone amide ^{1}H chemical shifts (Table S3) at $T = 36$ °C and in supercooled water provided evidence for the notion that the conformation of BPTI is, if at all, very little affected by the supercooling of the protein solution. This is consistent with an estimated cold denaturation melting temperature of about −40 °C or lower (see Supporting Information).

Flipping of Phe 45 (BPTI). To determine the flipping rate constant of Phe 45 (BPTI), k_{flip}(Phe 45), in supercooled water,

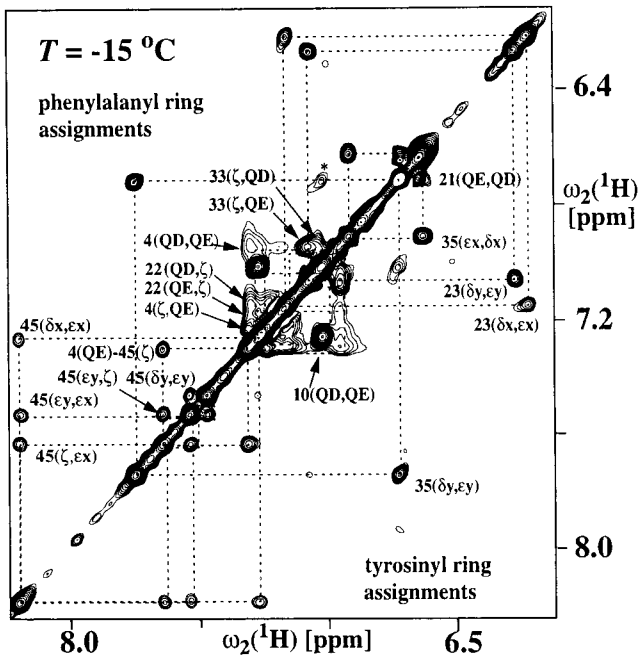

Figure 4. Spectral region comprising the aromatic resonances taken from a 2D [^{1}H,^{1}H]-NOESY spectrum ($\tau_{mix} = 40$ ms) recorded for BPTI at $T = -15$ °C. Cross-peak assignments of the phenylalanyl (above the diagonal) and the tyrosinyl rings (below the diagonal) (Table S1) are indicated. The time domain data were multiplied with a cosine function shifted by 45° in both dimensions. The asterisk labels a cross-peak arising from an impurity. Chemical shifts are relative to DSS.

we recorded cross-relaxation suppressed 2D [^{1}H,^{1}H]-EXSY spectra[18] (sample c1.0) from $T = -3.5$ to −16.5 °C (Figure 5). The resulting k_{flip}(Phe 45) values[23] (Table 1) are slightly smaller than those predicted for $T < 0$ °C (Figure 2). Because of the relatively small sub-zero temperature range (−3.5 °C $< T <$ −16.5 °C) covered by EXSY, highly accurate k_{flip}(Phe 45) values were required to obtain reliable activation parameters.[24] Our EXSY-derived rate constants (Table 1) were very close to those obtained from 2D NOESY as an independent body of experimental data (Figure S1) and yielded $\Delta H^{\ddagger} = 14 \pm 0.5$ kcal·mol^{-1} and $\Delta S^{\ddagger} = -4 \pm 1$ cal·mol^{-1}·K^{-1} (Figure 6).

The activation parameters were then compared with those derived from rate constants previously obtained[12] in the range 4 °C $< T <$ 72 °C (kindly provided by Dr. G. Wagner, Harvard Medical School, Boston, MA) when the same fitting protocol is employed, yielding $\Delta H^{\ddagger} = 16 \pm 1$ kcal·mol^{-1} and $\Delta S^{\ddagger} = 6 \pm 2$ cal·mol^{-1}·K^{-1}. The $\Delta H^{\ddagger}$ values determined in supercooled water and at ambient T are identical within their 2σ intervals, while the value for $\Delta S^{\ddagger}$ is slightly lower in the sub-zero temperature range. The significance of this finding has to be placed into the frame of the entire set of available activation parameters[12,14] (Figure 2). Remarkably, flipping of Phe 45 in BPTI is associated with the smallest so far known value for $\Delta S^{\ddagger}$, i.e., the other six well-characterized flipping rings of BPTI, Cytochrome *c* and Iso-2-cytochrome *c* exhibit $\Delta S^{\ddagger}$ values between 23 and 72 cal·mol^{-1}·K^{-1} (see legend of Figure 2). We

(23) To verify that the choice of a particular capillary diameter does not affect measurements, we recorded a supplementary series of 2D [^{1}H,^{1}H]-EXSY spectra with sample c1.7. The two values were identical within the experimental error (Table 1). Line shape analyses performed for the aromatic ^{1}H lines of Phe 45 above $T = 4$ °C were in excellent agreement with previous studies.[12] Hence, a significant impact on the ring-flipping rate potentially arising from the use of slightly different measurement conditions, when compared with previously published studies,[12] could also be excluded.

(24) Sandström, J. *Dynamic NMR Spectroscopy*; Academic Press: London, 1982.

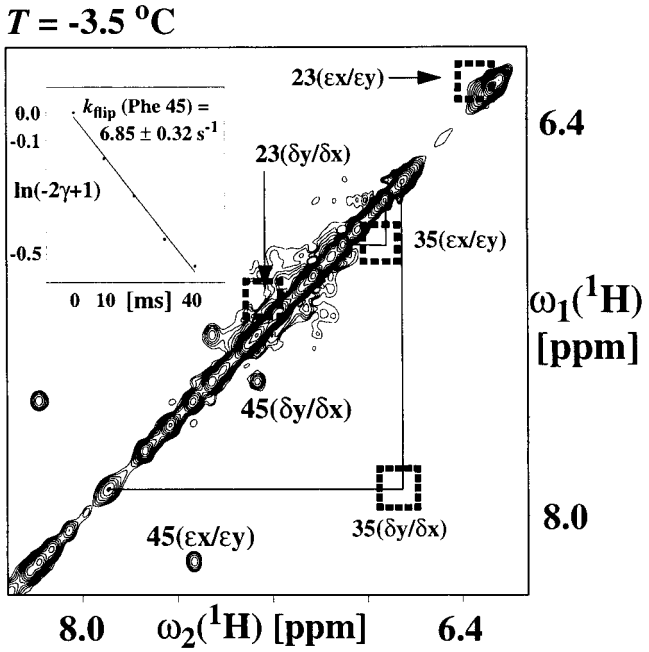

Figure 5. Spectral region containing the aromatic resonances taken from the cross-relaxation suppressed 2D [^{1}H,^{1}H]-EXSY[18] spectrum (τ_{mix} = 40 ms) acquired at $T = -3.5$ °C. The cross-peaks arising from the flip of Phe 45 are assigned ("ω_1/ω_2"), and the absence of cross-peaks for Tyr 35 and Tyr 23, which shows effective stalling of ring-flipping for these residues in supercooled water, are indicated by dashed boxes. Chemical shifts are relative to DSS. The insert shows the goodness of fit achieved for the determination of k_{flip}(Phe 45) from a series of such EXSY spectra recorded at different mixing times, τ_{mix}. The resulting value for k_{flip} is given. The experimental data were fitted to the equation $\ln(-2\gamma + 1) = -2k_{flip}\tau_{mix}$ (eq 1), where $\gamma = V_C/(V_C + V_D)$ and V_D and V_C represent the diagonal and cross-peak volumes, respectively, involving ^{1}H$^{\epsilon x/\epsilon y}$ of Phe 45. The experimental data were complemented with $\ln(-2\gamma + 1) = 0$ for $\tau_{mix} = 0$.

Table 1. Flipping Rate Constants of Phe 45 of BPTI Dissolved in Supercooled Water

	k_{flip} (Phe 45) [s^{-1}]	
temp [°C]	EXSY[a]	NOESY[b]
−3.0		∼8
−3.5	6.85 ± 0.32	
−5.0		∼6
−5.5	5.61 ± 0.32	
−7.0		∼5
−7.5	4.72 ± 0.22	
−9.0		∼4
−9.5[c]	3.70 ± 0.19	
−11.0		∼3.5
−12.5	2.75 ± 0.28	
−13.0		∼3
−14.5	2.23 ± 0.30	
−15.0		∼2.5
−16.5[d]	1.7 ± 0.7	

[a] From cross-relaxation suppressed EXSY.[18] [b] From NOESY.[10] [c] Averaged value obtained from two independent series of EXSY spectra acquired with samples c1.0 and c1.7 (see Materials and Methods). [d] Due to the larger experimental error, this value was not considered for determining activation parameters (Figure 7).

thus conclude that our measurements primarily demonstrate preservation of the so far uniquely small magnitude of $\Delta S^{\ddagger}$ that is associated with the flipping of the Phe 45 ring. Moreover, it has been assumed[12g] that both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are independent of T. While this assumption appears to be rather uncritical for the smaller temperature range studied here in supercooled water (−15 °C < T < −3.5 °C; Figure 6), it might possibly be that

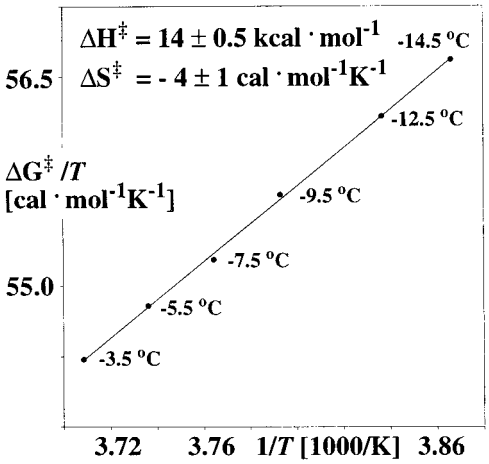

Figure 6. Eyring[15] plot for the ring-flip of Phe 45 (BPTI) in supercooled water derived with k_{flip} values determined in the range from $T = -14.5$ to −3.5 °C (Table 2) using cross-relaxation-suppressed 2D [^{1}H,^{1}H]-EXSY[18] (k_{flip} at −16.5 °C was not considered because of a much larger experimental uncertainty being due to the fact that the solutions contained in half of the capillaries were frozen at this T, see text). The resulting $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values are given. Their experimental error was estimated from (i) the standard deviations of the linear fit and (ii) considering a systematic error of 0.5 K for the measurement of the temperature. The goodness of the fit indicates that $\Delta H^{\ddagger}$ is, in a very good approximation, independent of T for the range studied here in supercooled water.

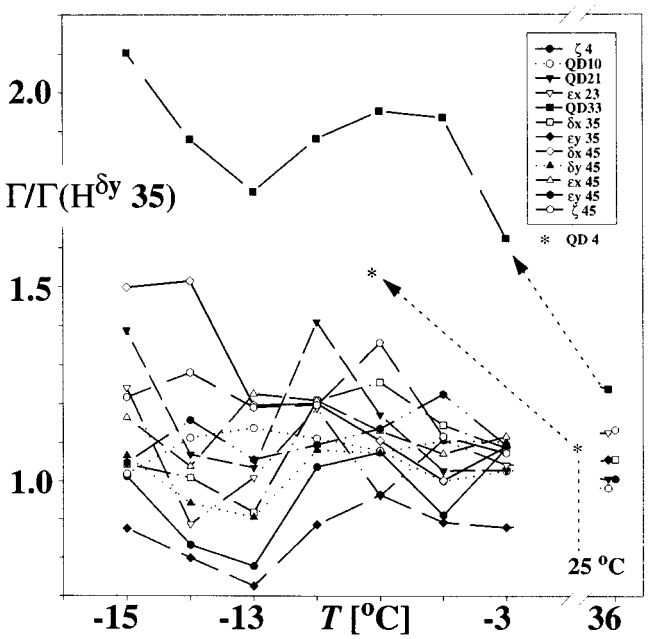

Figure 7. Aromatic ^{1}H line widths, Γ, of BPTI dissolved in supercooled water extracted from 2D [^{1}H,^{1}H]-NOESY. The ratios of the line widths of ring protons of Phe 4, Tyr 10, Tyr 21, Tyr 23, Phe 33, Tyr 35, and Phe 45 (labeled as defined *within* the box in the upper right) over the line width of ^{1}H$^{\delta y}$ of Tyr 35 are plotted versus the temperature, T. The line width of ^{1}H$^{\delta y}$ of Tyr 35 was chosen as a reference since its aromatic ring is not affected by flipping over the entire T-range from $T = 36$ to −16.5 °C (Figure 2). The line widths of QD of Phe 4 (marked with an asterisk) could not be measured in NOESY due to spectral overlap, and were thus obtained from 2D [^{13}C,^{1}H]-HSQC at the two temperatures 25 (indicated separately) and −6 °C.

a moderate change of $\Delta S^{\ddagger}$ in the range[12] 4 °C < T < 72 °C accounts for the observed difference.

Aromatic ring-flipping within the molecular core of a protein is intimately related to larger amplitude breathing modes of the

protein allowing the rings to flip.[12] Using $\Delta S^{\ddagger}$ as a physical−chemical parameter characterizing the distribution of larger amplitude motional modes connected to the flipping, the preservation of the outstandingly low $\Delta S^{\ddagger}$ value for Phe 45 in supercooled water indicates that the motional mode distribution associated with Phe 45 flipping in BPTI is only little affected by lowering T well below 0 °C. This suggests that, if a globular protein does not cold denature, aromatic flipping rates, and thus likely also the rates of other conformational and chemical processes occurring in supercooled water, can often be fairly well estimated from activation parameters obtained at ambient T.

Flipping of Tyr 23 and Tyr 35 (BPTI) in Supercooled Water. Activation parameters determined at ambient T predict (Figure 2) that ring-flipping of Tyr 23 and Tyr 35 is slowed to $k_{flip} < 0.3\ s^{-1}$ (Tyr 23) and $k_{flip} < 0.01\ s^{-1}$ (Tyr 35) for $T < -2$ °C, implying that associated ^1H and ^{13}C resonance line broadening[23] (i.e., $k_{flip}/\pi \ll 1$ Hz) is reduced beyond detection. An upper bound for the rate constants could be derived from the absence of cross-peaks in 2D EXSY[18] at $T = -3.5$ °C (Figure 5). In agreement with the predictions, we obtained $k_{flip} < 0.5\ s^{-1}$ at $T = -3.5$ °C for both rings.

Flipping of Phe 4, Tyr 10, Tyr 21, Phe 22, and Phe 33 (BPTI) in Supercooled Water. The complete assignment of the aromatic ^1H resonances down to $T = -15$ °C (Table S1) reveals that the rings of residues 4, 10, 21, 22, and 33 of BPTI are flipping fast on the chemical shift time scale, i.e., averaged $^1H^{\delta/\epsilon}$ chemical shifts are observed throughout (Figure 4). To evaluate if the flipping rates are reduced in supercooled water to an extent that aromatic ^1H NMR lines are beginning to be broadened in the fast exchange limit,[24] we measured the line widths in the range from $T = -3$ to -15 °C in 2D NOESY. Since $^1H^{\delta y}$ of Tyr 35 is not affected by ring-flipping (Figure 2) and shows well-resolved cross-peaks, its line width was used as a reference (Figures 2 and 4). Figure 7 shows the ratios of aromatic ^1H and $^1H^{\delta y}$(Tyr 35) line widths.

The data provide evidence for ring-flip induced broadening of QD and QE of Phe 33, and of QD of Phe 4 at sub-zero temperatures. For Phe 4, the resonance of QD is broadened in all spectra recorded in supercooled water (Figure 4) but is not broadened at $T = 25$ °C or higher. Peaks involving QE (and H^{ζ}) of Phe 4 are not affected in any of the spectra. This might well be due to the near-degeneracy of the two $^1H^{\epsilon}$ chemical shifts. Since no well-resolved cross-peaks were found for QD of Phe 4 in NOESY, we measured the ^1H line width in 2D [^{13}C,^1H]-HSQC (see below). The progressive line broadening observed for the Phe 4 and Phe 33 resonances in supercooled water suggests that these rings may flip slowly on the chemical shift time scale around $T = -25$ °C, a temperature which might be realized by designing capillary systems with smaller diameters.[3]

In contrast, the rings of Tyr 10, Tyr 21, and Phe 22 are either flipping fast enough to prevent detectable excess broadening even at $T = -15$ °C (Figure 7) or exhibit degenerate chemical shifts for both the H^{δ} and H^{ϵ} pairs when ring-flipping is stalled. Assuming (i) nondegenerate chemical shifts and (ii) that we could have detected a line broadening, $\Delta\nu_{broad}$, of about 10 Hz, lower bounds for the flipping rate can be estimated from BMRB data.[25] Considering that 95% of symmetry-related proton pairs with nondegenerate chemical shifts that are deposited in the BMRB[25] (see also Figure 10) exhibit chemical shift differences, $\Delta\delta$, between 0.05 and 1.4 ppm (i.e., $\Delta\nu$ between 38 and 1050 Hz at 750 MHz), the absence of broadening implicates a ~95% probability that the rate constants [approximated[24] using the relation $k_{flip} = \pi(\Delta\nu)^2/\Delta\nu_{broad}$] of Tyr 10, Tyr 21, and Phe 22 are still faster than the lower bounds ranging from 500 to $3 \times 10^5\ s^{-1}$ at $T = -15$ °C.[26]

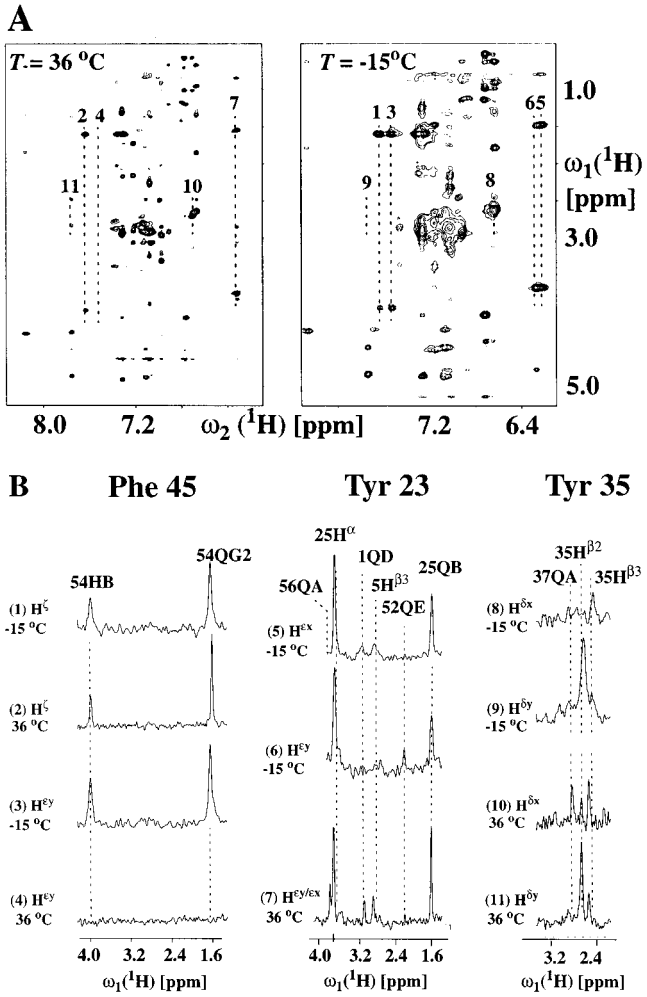

Figure 8. (A) Spectral regions comprising the aliphatic (ω_1)−aromatic (ω_2) NOEs taken from 2D [^1H, ^1H]-NOESY spectra recorded at $T =$ 36 °C ($\tau_{mix} = 70$ ms; on the left) and at (B) $T = -15$ °C ($\tau_{mix} = 15$ ms; on the right). The dotted lines indicate cross sections which are shown in part B. (B) Cross sections taken along ω_1 from the 2D [^1H,^1H]-NOESY spectra shown in part A. ("1") to ("4"), ("5") to ("7"), and ("8") to ("11") belong to Phe 45, Tyr 23, and Tyr 35, respectively, exhibiting decreasing k_{flip} in this order at ambient T.[12] The proton resonance assignment along ω_2 and the acquisition temperature are indicated on the left of each cross section. The data demonstrate that stalling of aromatic ring-flipping in supercooled water allows the collection of distinct sets of NOEs for the individual aromatic ^1H spins (see text).

Collection of Aromatic ^1H,^1H-NOEs (BPTI) in Supercooled Water. 2D NOESY spectra recorded at $T = -15$ and 36 °C were compared to demonstrate the impact of stalling aromatic ring-flipping for collection of NOEs. To ensure comparability of relative peak intensities,[10] the mixing times were scaled with $1/\tau_c$ (Figure 8).

At 36 °C, Tyr 23, Tyr 35, and Phe 45 flip rate constants have been measured[12g] to be between ~30 (Tyr 35) and ~1600 s^{-1} (Phe 45) (Figure 2). For the tyrosinyl rings, this leads to NOE averaging over H^{ϵ} and H^{δ} proton pairs and generation of conformational exchange affected[9,10] peaks, while the resonances of Phe 45 are broadened to an extent that detection of NOEs to $^1H^{\delta}$ or $^1H^{\epsilon}$ is impossible (Figure 8).

At −15 °C, the rings of Phe 45, Tyr 23, and Tyr 35 are flipping with k_{flip} being smaller than $\sim 2\ s^{-1}$ at $T = -15$ °C (Figures 5−7; Table 1). Hence, flipping of these rings is

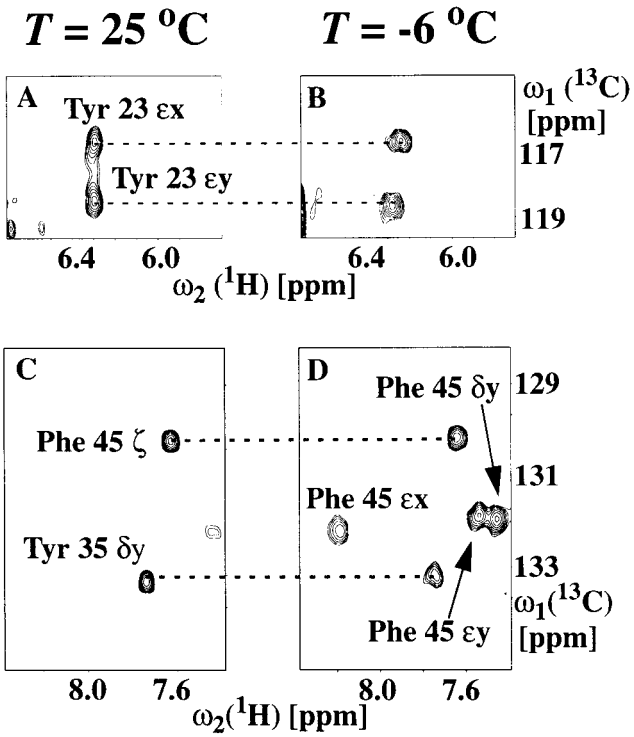

Figure 9. Contour plots of spectral regions taken from 2D [^{13}C,^{1}H]-HSQC comprising signals of the rings of Tyr 23 (A and B) and Phe 45 (C and D) at $T = 25$ (on the left) and -6 °C (on the right), exemplifying the use of 2D [^{13}C,^{1}H]-HSQC to assess the impact of supercooling on aromatic ring flips. (A, B) The dispersion of signals along ^{13}C allows resolving coincidental ^{1}H chemical shift degeneracy of the slowly flipping ring of Tyr 23 (see text). (C, D) The ϵ- and δ-resonances of Phe 45 are broadened nearly beyond detection at $T = 25$ °C, while the reduction of the flipping rate at $T = -6$ °C enables signal detection that is unperturbed by ring-flipping. Resonance assignments[12g] are indicated, and chemical shifts are given relative to DSS.[6]

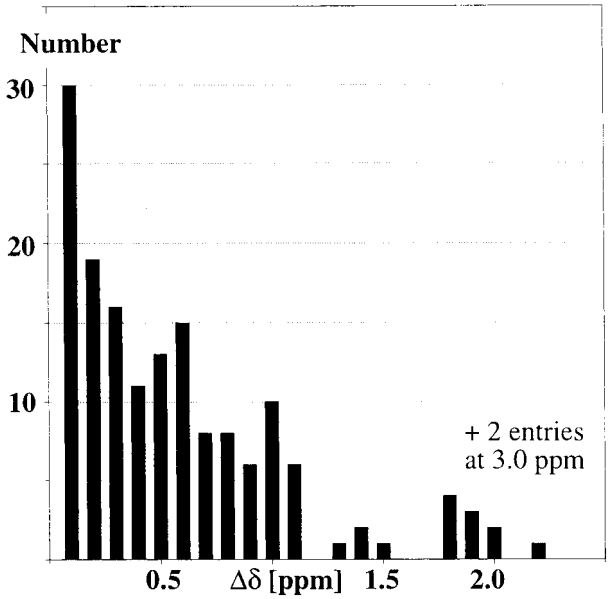

Figure 10. Distribution of chemical shift differences, $\Delta\delta$, for H^{ϵ} and H^{δ} pairs of Phe and Tyr rings with nondegenerate chemical shifts as deposited in the BioMagResBank.[25]

effectively stalled so that (i) NMR lines of the rings are no longer affected by the conformational exchange (in the slow exchange limit[24] $k_{flip} = 2\ s^{-1}$ translates into a broadening, k_{flip}/π, of <1 Hz) and (ii) NOE averaging is no longer observed ($k_{flip} = 2\ s^{-1}$ implies that less than 3% of the rings flip within 15 ms of mixing time; this suffices to generate detectable exchange cross-peaks derived from the most intense "diagonal magnetization", as observed for Phe 45 (Figures 5, S1), but prevents significant averaging of NOE cross-peaks). This allowed collection of distinct sets of NOEs being unperturbed by ring-flipping for the individual aromatic ^{1}H spins of Phe 45, Tyr 23 and Tyr 35 (Figure 8B).

Since H^{ζ} of Phe 45 is, if at all, only very little affected by ring-flipping, cross sections "1" and "2" of Figure 8B provide a reference to an aromatic resonance that is not affected by ring-flipping at both $T = -15$ and 36 °C. In sharp contrast, slices "3" and "4" show the impact of ring-flipping on the $^{1}H^{\epsilon y}$ resonance of Phe 45: no NOEs can be detected at 36 °C while strong signals are observed at -15 °C. The panels for Tyr 23 and Tyr 35 ("5" to "11" in Figure 8B) demonstrate how coincidental chemical shift degeneracy and/or fast flipping on the chemical shift time scale prevents assigning NOEs to the individual ring protons at the higher temperature and that supercooling affords a solution to circumvent this problem. A set of cross-peaks observed for both $^{1}H^{\epsilon y}$ and $^{1}H^{\epsilon x}$ of Tyr 23 at 36 °C can be distinctly assigned to either of the two ϵ-protons at -15 °C, e.g., the NOEs to QD of Arg 1, $H^{\beta 3}$ of Cys 5, and QE of Met 52 (compare "5" and "6" with "7" in Figure 8B). The $^{1}H^{\delta x}$ and $^{1}H^{\delta y}$ chemical shifts of Tyr 35 (Table S1) are well resolved at both $T = -15$ and 36 °C. Hence, the NOE-averaging registered at 36 °C is completely suppressed at -15 °C: cross-peaks involving $H^{\beta 2}$ and $H^{\beta 3}$ of Tyr 35 are observed for both $H^{\delta x}$ and $H^{\delta y}$ at 36 °C, but are detected only for either of the two aromatic protons in supercooled water at $T = -15$ °C (compare "8" and "9" with "10" and "11", respectively, in Figure 8B).

2D [^{13}C,^{1}H]-HSQC (BPTI) in Supercooled Water. For ^{13}C-labeled proteins, 2D [^{13}C,^{1}H]-HSQC will be the experiment of choice to rapidly investigate the impact of supercooling on aromatic ring-flipping (see also Figure 3 in ref 12 g). At $T = 25$ °C, the ring of Phe 45 is flipping in the intermediate regime on the ^{1}H chemical shift time scale (Figure 2) and ^{1}H lines are nearly broadened beyond detection. In sharp contrast, supercooling of the protein solution enables observation of NMR lines hardly affected by ring-flipping (see above). This is well

(25) The search of the BioMagResBank (http://www.bmrb.wisc.edu) alluded to in this publication was conducted on July 15, 2000. Chemical shift differences smaller than 0.05 ppm were considered as degenerate (22 and 23 entries for Tyr and Phe, respectively). Since the chemical shift differences of the H^{ϵ} and H^{δ} pairs are not related to the chemical nature of the ring but are due to the anisotropic environment within the protein, identical average differences were found for Tyr (0.50 ± 0.51 ppm) and Phe (0.43 ± 0.56 ppm). The corresponding distribution of chemical shift differences (Figure 10) can be recruited to derive bounds for flipping rate constants from detected line broadening. The average temperature, $\bar{T}$, of all entries of the BMRB is $\bar{T} = 29 \pm 11$ °C, and for entries with nondegenerate proton pairs $\bar{T} = 28 \pm 10$ °C for Tyr and $\bar{T} = 31 \pm 13$ °C for Phe. Notably, the T-distribution (Figure S2) at which aromatic ^{1}H chemical shifts deposited in the BMRB were measured is quite similar for nondegenerate chemical shift pairs and the entire BMRB, i.e., the selection for nondegeneracy is not detectably correlated with a selection for reduced T. For larger proteins one might expect to encounter an increasing number of rings being held in a fixed orientation within the molecular core. However, no statistically significant correlation could be detected between the frequency of nondegenerate chemical shift pairs and the proteins' molecular weights. Although it might well be that the current number of BMRB entries is still too small to derive statistically significant insights, we are left with tentative support of the notion (inferred from calculation of solvent acessibilities) that the free activation enthalpies of larger amplitude modes enabling ring-flipping might well be of comparable magnitude when involving either primarily the molecular core or more peripheral segments.

(26) Similarly, we can expect with ~95% probability that the ring of Phe 33, for which an excess line broadening of about 20 Hz is registered for the QD resonance at $T = -15$ °C, is flipping with a rate constant in the range $250\ s^{-1} < k_{flip} < 10^{5}\ s^{-1}$ at this temperature.

documented for Phe 45 and Tyr 23 in 2D [^{13}C,^{1}H]-HSQC spectra recorded at natural ^{13}C isotope abundance (Figure 9). Furthermore, 3D ^{13}C-resolved NOESY promises to break coincidental ^{1}H chemical shift degeneracy of slowly flipping Tyr and Phe rings, as it is neatly exemplified for the CH$^{\delta}$ resonances of Tyr 23 (Figure 9).

Aromatic Ring Flips and Solvent Accessibility. It has been suggested that slowed ring-flipping correlates with burial in the proteins' molecular cores.[12] We calculated the solvent accessibilities of the aromatic rings of BPTI, Cytochrome *c* (Figure 1), and Iso-2-cytochrome *c* from their high-resolution X-ray structures.[27] These calculations revealed no readily apparent correlation of flip rates and solvent accessibility, i.e., the most slowly flipping rings are among both the buried ones (e.g., Tyr 35 of BPTI) as well as the more exposed ones (e.g., Tyr 97 of Iso-2-cytochrome *c*). In particular, the ring of Phe 33 of BPTI is virtually completely buried but flips rapidly on the chemical shift time scale even at $T = -15$ °C (Figures 4 and 7). This suggest that, at least for smaller globular proteins, solvent accessibility is, after all, not a key parameter for identifying slowly flipping rings. Clearly, it is the particular nature of the larger amplitude motional mode allowing the ring to flip which determines the associated free activation enthalpy. Hence, we are left with the impression that the activation parameters of such larger amplitude modes may well be of comparable magnitude when involving either primarily the molecular core or more peripheral segments of BPTI. Intriguingly, this scenario contradicts the more often intuitively adopted view that the molecular core represents a "more rigid" part of a protein.

Search of the BioMagResBank. Nondegeneracy of H$^{\epsilon}$ and H$^{\delta}$ shifts[6] indicates a ring-flipping process that is slow on the chemical shift time scale. Assuming that only a small fraction of H$^{\epsilon}$ and H$^{\delta}$ chemical shift pairs exhibit coincidental chemical shift degeneracy, the fraction of nondegenerate pairs in the BioMagResBank (BMRB)[25] should be a valuable measure for the fraction of Tyr and Phe rings that are slowly reorienting on the shift time scale at ambient *T*. A search of BMRB revealed that (i) 121 out of the 3213 tyrosinyl H$^{\epsilon}$ and H$^{\delta}$ chemical shift pairs, i.e. 3.8%, and (ii) 49 out of the 3175 phenylalanyl pairs, i.e. 1.5%, are nondegenerate. Even when considering that a fraction of the rings could not be assigned because of flip-broadened lines, we are left with the intriguing conclusion that the overwhelming majority of aromatic Tyr and Phe rings are flipping rapidly at ambient *T*. Further recognizing that in BPTI and Iso-2-cytochrome *c* we find that six out of seventeen rings (~35%) are effectively "frozen in" below −10 °C, this may suggest that a large fraction of the Tyr and Phe rings flipping rapidly at ambient *T* may be slowed in supercooled water.

Conclusions

The present investigation shows that the solvent "supercooled water" does per se not affect protein structure or dynamics, which thus allows one to conduct NMR-based structural biology at sub-zero temperature in an unperturbed aqueous environment. This conclusion is in good agreement with our previous study of the 9 kDa protein ubiquitin.[2]

Currently known activation parameters (Figure 2) indicate that Tyr rings are more likely to flip slowly than Phe rings, a view that is supported by our search of the BMRB.[25] Furthermore, relatively similar activation parameters were measured for the Tyr rings flipping slowly in BPTI, Iso-2-cytochrome *c*, and Cytochrome *c* (Figure 2), and they are quite different for the only Phe ring for which these parameters have been published. It remains to be seen whether this observation, which actually indicates that many of the Tyr rings flipping more slowly at ambient *T* can be effectively "frozen in" at readily attainable *T* around −10 °C, is of more general nature.[30,31] Moreover, the majority of Phe and Tyr rings are rapidly flipping at ambient *T* and thus bear the potential to be brought to intermediate to slow exchange on the chemical shift time scale. Hence, supercooling of protein solutions promises to harvest the potential benefits of stalled ring-flipping for refining NMR solution structures,[2,11] to recruit additional aromatic rings for investigating protein dynamics, and to use multiple slowly flipping rings to probe cold denaturation.

Protocols ensuring efficient protein deuteration,[33] the introduction of the transverse relaxation optimized spectroscopy (TROSY) detection scheme,[34] and the recruitment of residual dipolar couplings[35] have not only expanded the use of NMR to larger systems, but they also promise increased accuracy for NMR structures of smaller biological macromolecules.[36] These novel approaches are, however, not tailored to meet with a major caveat for the generation of ultrahigh-quality NMR structures: *internal mobility*. Protein deuteration and TROSY can relax on constraints imposed by longer correlation times of the overall rotational reorientation, but cannot alleviate the impact of conformational exchange broadening or ring-flip associated NOE averaging. In contrast to spin diffusion, such NOE averaging can also not be efficiently suppressed by shortening the mixing time,[10,11] so that a suitable reduction of *T* appears to be the approach of choice. We thus conclude that NMR of supercooled protein solutions may develop into a valuable complement to the current arsenal of methods for NMR structure refinement.[2]

(27) Solvent accessibilities of the aromatic rings of tyrosinyl and phenylalanyl residues were calculated for BPTI,[28a] Iso-2-cytochrome *c*[28b] and Cytochrome *c*[28c] from corresponding X-ray structures[28] using the program Molmol.[29] The following ring accessibilities were obtained. BPTI: Phe 4 (25%), Phe 22 (21%), Phe 33 (2%), Phe 45 (15%), Tyr 10 (31%), Tyr 21 (20%), Tyr 23 (5%), Tyr 35 (5%). Iso-2-cytochrome *c*: Phe(−3) (17%), Phe 10 (10%), Phe 36 (1%), Phe 82 (16%), Tyr 46 (14%), Tyr 48 (17%), Tyr 67 (8%), Tyr 74 (15%), Tyr 97 (17%). Cytochrome *c*: Phe 10 (12%), Phe 36 (6%), Tyr 46 (16%), Phe 82 (24%), Tyr 48 (14%), Tyr 67 (9%), Tyr 74 (11%), Tyr 97 (8%). Residues with rings for which the Eyring activation parameters[19] were determined[12,14] are underlined.

(28) (a) Wlodawer, A.; Nachman, J.; Gilliland, G. L.; Gallagher, W.; Woodward, C. *J. Mol. Biol.* **1987**, *198*, 469−480. (b) McGee, W. A.; Rosell, F. I.; Liggins, J. R.; Rodriguez-Ghidarpour, S.; Luo, Y.; Chen, J.; Brayer, G. D.; Mauk, A. G.; Nall, B. T. *Biochemistry* **1996**, *35*, 1995−2007. (c) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595.

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(30) Notably, a very recent high-pressure study of ring-flipping in BPTI[30] yielded rather accurate activation volumes[12e] of 85 ± 20 and 46 ± 9 Å^3 for Tyr 35 and Phe 45, respectively. Assuming that the motional modes associated with the ring-flipping are occurring in the low-viscosity limit,[12g,31] these values indicate that a larger cavity is required for a Tyr ring to flip when compared with Phe. One may then speculate that, on average, the formation of a large cavity is connected to a larger free activation enthalpy. This would then be consistent with the rings of Tyr being twice as often found[25] to flip slowly at ambient *T* when compared with Phe (Figure S2).

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The accurate determination of the activation parameters characterizing the ring flip of Phe 45 in supercooled water and subsequent comparison with those obtained at ambient *T* provided novel insight into the *T*-dependence of larger amplitude motional mode distributions of BPTI, i.e., their robustness with respect to approaching very low *T*. Considering the sensitivity of ring-flipping rates to changes in global protein dynamics, we anticipate that studying these flips in supercooled water will also become a valuable novel approach to detect the onset of cold denaturation. For a protein cold denaturing with a melting temperature around $T = -20$ °C, one could expect to register reduced ring-flipping rates when entering the sub-zero temperature range. At further reduced *T*, *increasing* rates arising from the onset of cold denaturation (i.e., a pre-transition change in dynamics), and the associated introduction of new larger amplitude motional modes can be anticipated.

Finally, the ever increasing performance of supercomputers will propel molecular dynamics simulations of globular proteins into the range of microseconds[37] or longer. One may thus envisage that aromatic ring-flipping, which has been a keen topic for theoreticians in the late 1970s and 1980s,[38] will again attract interest to effectively calibrate force fields. Routine employment ^{13}C-resolved EXSY[10,39] (or TROSY[34] versions thereof) for ^{13}C-labeled proteins dissolved in supercooled water promises to largely increase the number of aromatic rings for which flipping rate constants, and possibly activation parameters, are measured in the future. In the longer run, this may also support further improved protein fold prediction protocols, thereby constituting another paradigm for the impact of NMR spectroscopy for structural genomics.[40]

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Supporting Information Available: Extended description of Materials and Methods; Tables S1, S2, and S3 with aromatic ^{1}H, methyl ^{1}H/^{13}C, and backbone amide ^{1}H chemical shifts, respectively; estimation of BPTI's cold denaturation melting temperature; and Figures S1 and S2 showing cross sections from 2D [^{1}H,^{1}H]-NOESY between $T = -3$ and -15 °C and the *T*-distribution for aromatic proton chemical shift entries deposited in the BioMagResBank, respectively (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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