On the origins of apparent fragile-to-strong transitions of protein hydration waters

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$^2$H NMR is used to study the mechanisms for the reorientation of protein hydration water. In the past, crossovers in temperature-dependent correlation times were reported at $T_{x1} \approx 225 \text{ K (X1)}$ and $T_{x2} \approx 200 \text{ K (X2)}$. We show that neither $X_1$ nor $X_2$ are related to a fragile-to-strong transition. Our results rule out an existence of $X_1$. Also, they indicate that water performs thermally activated and distorted tetrahedral jumps at $T < T_{x2}$, implying that $X_2$ originates in an onset of this motion, which may be related to a universal defect diffusion in materials with defined hydrogen-bond networks.

Studying the temperature dependence of the structural relaxation, one can distinguish 'strong' and 'fragile' glass-forming liquids, which do and do not show Arrhenius behavior, respectively [1]. For supercooled bulk water, it was proposed that a fragile-to-strong transition (FST) exists at about $225 \text{ K}$ [2,3]. However, inevitable crystallization interferes with direct observation [4]. In confinement, crystallization can be avoided so that the dynamics of supercooled water are accessible down to the glass transition temperature $T_g$. For confined water, crossovers in the $T$ dependence of a correlation time $\tau$ were observed at $T_{x1} \approx 225 \text{ K (X1)}$ and $T_{x2} \approx 200 \text{ K (X2)}$ using quasi-elastic neutron scattering (QENS) [5,6] and dielectric spectroscopy (DS) [7,8], respectively. Several workers took $X_1$ and $X_2$ as indications for a FST [3,8]. Also, $X_1$ was related to a liquid-liquid phase transition [9,10]. Challenging these conclusions, others argued that the Arrhenius processes $P_1$ and $P_2$ observed below $X_1$ and $X_2$, respectively, are secondary ($\beta$) relaxations [11,12], while structural ($\alpha$) relaxation is difficult to observe [8].

Confined waters are of enormous importance for biological, geological, and technological processes, e.g., an interplay of water and protein dynamics enables biological functions [13]. Here, we investigate the hydration waters of elastin (E) and collagen (C), two proteins of the connective tissue. Using $^2$H NMR, we study time scale and mechanism of water reorientation during $T_{x1}$ and $T_{x2}$. The results show that a FST does not exist, while tetrahedral jumps become important upon cooling.

In $^2$H NMR, the quadrupolar frequency is probed [11]:

$$\omega_Q(\theta, \phi) = \pm \frac{\delta}{2} (3 \cos^2 \theta - 1 - \eta \sin^2 \theta \cos 2\phi)$$

(1)

Here, the angles $(\theta, \phi)$ describe the orientation of the electric field gradient (EFG) tensor at the nuclear site with respect to the external static magnetic field. Since the molecular orientation determines the orientation of the EFG tensor, rotational jumps render $\omega_Q$ time dependent. The anisotropy and asymmetry of the tensorial interaction are characterized by $\delta$ and $\eta$, respectively. The $\pm$ signs correspond to two allowed transitions between three Zeeman levels of the $^2$H nucleus ($I=1$).

C, E, and D$_2$O were purchased from Aldrich. Weighed amounts of protein and D$_2$O were carefully mixed and sealed in the NMR tube to prepare samples with hydration levels $h = 0.25$–0.96 (g D$_2$O/1 g protein). We refer to the samples as ‘E’ or ‘C’ followed by the value of $h$ in percent. $^2$H NMR spin-lattice relaxation (SLR), line-shape (LS), and stimulated-echo (STE) measurements are performed at a Larmor frequency of $\omega_L = 2\pi \times 76.8 \text{ MHz}$. The experimental setup is described in Ref. [15].

In the studied samples, supercoolable bound water and freezable free water can coexist. Furthermore, deuterons replace exchangeable hydrogens of the protein [16]. On the basis of the amino acid compositions [17], one expects 10–30% of the deuterons to be bound to proteins for the used hydration levels. Hence, deuterons of supercooled water, crystalline water, and protein can contribute to $^2$H NMR signals. Accordingly, three steps are observed in SLR measurements for C60, see Fig. 1(a). The third step results from deuterons of crystalline water, as can be inferred from its absence for low hydration levels, e.g., for C25, when free water does not exist [18,19,20]. Since water deuterons outnumber protein deuterons, the higher first and lower second steps can be assigned to supercooled water and protein, respectively. The existence of distinct steps means that deuteron exchange between the three species is slow on the time scale of SLR [10], enabling separate analysis of the dynamical behaviors in $^2$H.
NMR.

Depending on the value of $h$, we use two- or three-step relaxations to fit the buildup of magnetization, $M_z(t)$, see Fig. (1a). While we will not discuss results for crystalline water, Figure (2a) compares the $T$-dependent SLR times $T_{1w}$ and $T_{1p}$ of deuterons in supercooled waters and proteins, respectively. $T_{1w}$ shows a very similar minimum for all samples, indicating that water dynamics in the hydration shells of C and E are highly comparable. Unlike the first step, the second step is nonexponential ($\beta_p \approx 0.6$), typical of amorphous solids [14]. For C and E, $T_{1p}$ and, thus, the protein dynamics weakly depend on $T$.

Using that reorientation of the hydration waters is basically isotropic in the vicinity of the minimum, see below, $T_{1w}$ depends on the spectral density according to [14]

$$T_{1w}^{-1} = \frac{2}{15} \delta^2 \left[ J(\omega_L) + 4J(2\omega_L) \right]$$

(2)

Here, $J(\omega) = \int_0^\infty F_2(t) \cos(\omega t) dt$. The rotational correlation function (RCF) $F_2(t) \propto \langle P_2(\cos(\theta)) \rangle^2$ describes the time dependence of the Legendre polynomial $P_2$ of the angle $\theta$. For a Debye process, $F_2(t) = \exp(-t/\tau)$ and $J_{BP P}(\omega) = \tau/(1+\omega^2\tau^2)$. In this case, Eq. (2) takes the form derived by Bloembergen, Purcell, and Pound (BPP) [21]. However, this approach predicts $T_{1w} = 2.4$ ms at the minimum, in variance with our results in Fig. 2(a).

Thus, a Debye process does not describe the water dynamics at the minimum, at variance with our results in Fig. 2(a). The assignment and rephasing periods $\Delta$ of the solid-echo sequence interfere with echo formation [23]. Random-walk simulations [24] for various motional models show that $T_{1w}$, found in QENS works below $185 \text{ K}$, is time-averaged $M(z)$ after saturation, but start acquisition at times $\tau_{p1}$ found in QENS works below $T_{1w}$ (Fig. 3c), in clear contrast to the findings in Fig. 1(b). The assignment and shape of the lines are confirmed when we single out the water contribution in partially relaxed (PR) experiments [14]. For the water component, this LS is observed at low T, but the broad spectrum collapses between 185 and 215 K resulting in a narrow Lorentzian at high T. The narrow spectrum reveals that, at $T \geq 215 \text{ K}$, the water molecules show fast ($\tau < 1/\delta$) isotropic jumps that average out the anisotropy of the quadrupolar interaction, while anisotropic motions, e.g., $\pi$ flips, do not result in a Lorentzian and can be excluded. At $T \leq 185 \text{ K}$, the broad spectrum indicates that significant water dynamics is absent on the time scale of $1/\delta \approx 1 \mu s$. Thus, $P_{1f}$, found in QENS works below $185 \text{ K}$, is not the $\alpha$ process so that $X_1$ is not a FST. If $P_{1f}$ were the $\alpha$ process, a narrow Lorentzian would be observed as $^2$H LS down to about $140 \text{ K}$ due to $\tau_{p1} < 1/\delta$, see Fig. 3(c), in clear contrast to the findings in Fig. 1(b). The assignment and shape of the lines are confirmed when we single out the water contribution in partially relaxed (PR) experiments (not shown), in which we do not wait for complete recovery of $M_z$ after saturation, but start acquisition at times between first and second SLR steps.

The total spectral intensity $S$, determined by integrating the solid-echo spectra after correction for the Curie factor, exhibits a minimum $S \approx 0.25$ at $T \approx 210 \text{ K}$, see Fig. 2(b), indicating that water dynamics during the dephasing and rephasing periods $\Delta$ of the solid-echo sequence interferes with echo formation [23]. Random-walk simulations [24] for various motional models show that $S$ is a minimum at $\tau = 3 \mu s$. The signal is not reduced for much faster (slower) dynamics, when $\omega Q$ is time-averaged (time-invariant). Deviations between measured and calculated data result because the simulations do not con-
FIG. 3: (a) $F_2^w(t_m; t_p = 30 \mu s)$ of E43 at various $T$ and fits to Eq. (3). (b) $F_2^w(t_m; t_p = 2, 10, 40 \mu s)$ of E30 from PR experiments at 185 K and fits to Eq. (4). (c) Correlation times from LS (triangle) and SLR analysis at $T \geq 195$ K ($JC_D$: circles and squares, $JCC$: stars) and from STE analysis below 195 K [E43: $\langle \tau_{ss} \rangle$ from $F_2^w(t_m; t_p = 30 \mu s)$, E30: $\langle \tau_{ss} \rangle$ from PR $F_2^w(t_m; t_p = 2 \mu s) \approx F_2^w(t_m)$]. Open symbols mark differing SLR results from $JC_D$ and $JCC$. Correlation times from measurements using DS (solid line) hydrated myoglobin [33] and (dotted line) E $(h=0.1)$ [19], (+) TSC for E $(h=0.5)$ [20]. (×) MR for C $(h \geq 0.5)$ [18], and (□) QENS for hydrated lysozyme [19]. (d) Evolution-time dependence of $\langle \tau_{cc} \rangle$ (solid circles) and $\langle \tau_{ss} \rangle$ (open circles) from PR STE experiments on E30 at 185 K and simulation results for (+) distorted $(\pm 3^\circ)$ tetrahedral jumps and (×) isotropic $10^\circ$ jumps.

R$_p$ from the above SLR analysis, we fit $F_2^w(t_m)$ to

$$A_w[(1-B)\Phi_w(t_m)+B R_w(t_m)+(1-A_w)R_p(t_m)]$$

Here, $B$ is introduced to consider that water dynamics does not destroy all orientational correlation, see below. Using a stretched exponential $\Phi_w(t_m) = \exp[-(t_m/\tau)^{\beta}]$, we obtain stretching parameters $\beta = 0.27 - 0.28$ [22] for all studied values of $t_p$ and $T$. Thus, hydration water exhibits strongly nonexponential RCF below 200 K. Use of the $\Gamma$ function enables calculation of the mean correlation time, $\langle \tau \rangle = (\tau/\beta)\Gamma(1/\beta)$. In Fig. 3(c), we see that $\langle \tau_{ss} \rangle$ and $\langle \tau_{cc} \rangle$ from $F_2^w$ of E30 and $F_2^w$ of E43, respectively, follow the same Arrhenius law (dashed line) with activation energy $E_a = 0.45 eV$. We note that $F_2^w$ of E30 was obtained in PR experiments to minimize the protein contribution, leading to $(1-A_w) = 0.05 - 0.10$.

Comparison of our SLR, LS, and STE data implies a crossover in the $T$ dependence of water dynamics in the vicinity of $T_{c2}$. Here, exact determination of a crossover temperature is hampered by a dependence on the choice of the spectral density used in SLR analysis. For an understanding of the origin of $X_2$, knowledge about the mechanism for water dynamics below $T_{c2}$ is of particular importance. We expect that the exploitation of STE decays on the length of the evolution time has been shown [14, 20] to yield valuable insights into the geometry of rotational motion since sensitivity to small changes of $\omega_Q$ and, thus, small angular displacements is higher for large $t_p$, see Eq. (4). An analysis of $\langle \tau_x(t_p) \rangle$ enables a determination of jump angles. While small-angle reorientation, results in a strong decrease of $\langle \tau_x(t_p) \rangle$, $\langle \tau_x \rangle$ is independent of $t_p$ for large-angle reorientation [14], see Fig. 4. Extraction of $\langle \tau_x(t_p) \rangle$ from $F_2^w(t_m; t_p)$ of E30 shows that a substantial dependence on $t_p$ is absent, in harmony with results for C25 (not shown). Hence, below $T_{c2}$, water exhibits large-angle rather than small-angle reorientation typical of the $\alpha$ process [14]. In Fig. 4(b), we see that $F_2^w(t_m; t_p)$ decays to a small, but finite plateau $B(t_p)$, see Eq. (1), before the onset of SLR. The plateau height depends on the geometry of the motion, e.g., $B(t_p \to \infty) = 1/n$ for a $n$-site jump [20]. Here, $B$ decreases from 0.16 to 0.06 when $t_p$ is extended from 2 to 40 $\mu s$, indicating that, though water reorientation is not isotropic, angular restrictions are not severe [20]. While exact two- ($B = 1/2$) or four-site ($B = 1/4$) jumps can be excluded, distorted tetrahedral jumps, which may be expected for a disordered hydrogen-bond network, are consistent with the data [20]. In $\text{^2H}$ STE work on ice $I_b$ [27], a similar dependence $B(t_p)$ was shown to indicate that translational diffusion of water molecules via interstitial defects involves distorted $(\pm 3^\circ)$ large-angle reorientation between 7 O-D bond orientations in the crystal.

In summary, we exploited the capabilities of $\text{^2H}$ NMR to ascertain correlation times and mechanisms for the rotational motion of supercooled protein hydration waters.
In the literature, it was a controversial issue to take crossovers $X_1$ and $X_2$ of temperature-dependent $\tau$ as evidence for a FST. Prerequisite for such interpretation of $X_1$ or $X_2$ is that the processes $P_1$ or $P_2$ below these crossovers are the $\alpha$ process. The present results, e.g., the LS at 145–185 K, rule out that $P_1$ is the $\alpha$ process and, hence, that $X_1$ at $T_{x1}\approx 225$ K is a FST. While a narrow spectrum would result if $P_1$ were the $\alpha$ process, a broad spectrum is observed. Our findings conflict with QENS [6, 10] and $^1$H NMR diffusion [28] studies, attributing $X_1$ to a FST. Further evidence against $X_1$ being a FST comes from extrapolation of $\tau_{P_1}(T)$, which yields too small values $T_\alpha < 100$ K. Also, a weak wavevector dependence $\tau(Q)$ below $X_1$ [6] implies local rather than diffusive motion. Our results are consistent with the conjecture that $P_1$ is a $\beta$ process [8,11,12], if the underlying motion is too restricted to be probed in $^2$H NMR.

Exploiting the sensitivity of $^2$H NMR to the mechanisms for water reorientation, we investigated the origin of $X_2$ at $T_{x2} \approx 200$ K, reported in DS work [8,11]. Above $T_{x2}$, SLR and LS analyses showed that water exhibits isotropic reorientation described by a broad distribution $G(\log \tau)$. Below $T_{x2}$, STE experiments indicate that water performs large-angle jumps, most probably distorted tetrahedral jumps, which follow an Arrhenius law with $E_a = 0.45$ eV. For $P_2$, DS work on water in various confinements [11] reported comparable $\tau_{P_2}(T)$, see Fig. 3(c). Also, mechanical-relaxation (MR) and thermally-stimulated currents (TSC) studies found a process with similar values of $\tau$ [18, 20]. Therefore, all these methods probe $P_2$. However, $P_2$ neither destroys all orientational correlation, see Fig. 3 nor affects rigidity [18]. Thus, $P_2$ is not the $\alpha$ process and $X_2$ is not a FST. Moreover, the large-angle jump mechanism is strong evidence against $P_2$ being a Johari-Goldstein $\beta$ process [29], which results from small-amplitude reorientation [30]. Comparison with previous $^2$H NMR work [23] showed that diffusion of water molecules via interstitial defects is consistent with the present results. Since $E_a \approx 0.45$ eV is not only found for water in various confinements [11], but also for crystalline and glassy bulk water [8], we suggest that water shows a characteristic tetrahedral jump motion, which is controlled by breaking of hydrogen bonds and, possibly, related to interstitial defect diffusion whenever a defined hydrogen-bond network is established, although we cannot exclude that the tetrahedral jump motion is governed by the protein surfaces in our case. The onset of this tetrahedral jump motion leads to more or less pronounced crossovers $X_2$. Extrapolating $\tau_{P_2}(T)$, a value of 100 s is reached in the vicinity of 136 K, the first widely accepted [6], but later questioned [6] value of $T_\alpha$ for bulk water. Thus, one might speculate that the reported small calorimetric effects are not related to a glass transition, but to freezing of interstitial defects.

$^2$H NMR, which probes single-particle RCF, cannot resolve the issue whether the $\alpha$ process exists below $X_2$, because $P_2$ destroys basically all orientational correlation before an onset of structural relaxation. Detection of the $\alpha$ process rather requires techniques sensitive to the reorganisation of the whole hydrogen-bond network. In this respect, it is interesting that MR and TSC studies [18, 20] found a slower process $P_3$, see Fig. 3(c), which affects rigidity and, hence, may be the $\alpha$ process. Finally, it is an open question whether the onset of tetrahedral jump motion is related to a liquid-liquid phase transition, which was proposed to lead to a low-density liquid with a more ordered tetrahedral network upon cooling [8, 4].

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