Observation of Fragile-to-Strong Dynamic Crossover in Protein Hydration Water

Sow-Hsin Chen, Li Liu, Emiliano Fratini, Piero Baglioni, Antonio Faroone, and Eugene Mamontov
1Department of Nuclear Science and Engineering, Massachusetts Institute of Technology, Cambridge MA 02139 USA 2Department of Chemistry and CSGI, University of Florence, via della Lastruccia 3, 50019 Florence, Italy 3Department of Material Science and Engineering, University of Maryland, College Park, MD 20742 USA and NIST Center for Neutron Research, Gaithersburg, MD 20899-8562 USA

At low temperatures proteins exist in a glassy state, a state which has no conformational flexibility and shows no biological functions. In a hydrated protein, at and above 220 K, this flexibility is restored and the protein is able to sample more conformational sub-states, thus becomes biologically functional. This 'dynamical' transition of protein is believed to be triggered by its strong coupling with the hydration water, which also shows a similar dynamic transition. Here we demonstrate experimentally that this sudden switch in dynamic behavior of the hydration water on lysozyme occurs precisely at 220 K and can be described as a Fragile-to-Strong dynamic crossover (FSC). At FSC, the structure of hydration water makes a transition from predominantly high-density (more fluid state) to low-density (less fluid state) forms derived from existence of the second critical point at an elevated pressure.

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Without water, a biological system would not function. Dehydrated enzymes are not active, but a single layer of water surrounding them restores their activity. It has been shown that the enzymatic activity of proteins depends crucially on the presence of at least a minimum amount of solvent water [1, 2]. It is believed that about 0.3 g of water per g of protein is sufficient to cover most of the protein surface with a single layer of water molecules and to fully activate the protein functionality. Thus, biological functions [3], such as enzyme catalysis, can only be understood with a precise knowledge of the behavior of this single layer of water and how that water affects conformation and dynamics of the protein. The knowledge of the structure and dynamics of water molecules in the so-called hydration layer surrounding proteins is, therefore, of utmost relevance to the understanding of the protein functionality. It is well documented that at low temperatures proteins exist in a glassy state [4, 5], which is a solid-like structure without conformational flexibility. As the temperature is increased, the atomic motional amplitude increases linearly initially, as in a harmonic solid. In hydrated proteins, at approximately 220 K, the rate of the amplitude increase suddenly becomes enhanced, signalling the onset of additional anharmonic and liquid-like motion [6, 7, 8, 9]. This 'dynamical' transition of proteins is believed to be triggered by their strong coupling with the hydration water through the hydrogen bonding. The reasoning is derived from the finding that the protein hydration water shows some kind of dynamic transition at the similar temperature [10, 11]. Here we demonstrate, through a high-resolution quasi-elastic neutron scattering (QENS) experiment, that this dynamic transition of hydration water on lysozyme protein is in fact the Fragile-to-Strong dynamic crossover (FSC) at 220 K, similar to that recently observed in confined water in cylindrical nanopores of silica materials [12, 13]. Computer simulations on both bulk water [14] and protein hydration water around lysozyme [15] have led to the interpretation of the FSC as arising from crossing the locus of maximum in the correlation length ("Widom line") which emanates from a critical point into the one-phase region: if this interpretation is correct, then our experiments provide evidence supporting the existence of a liquid-liquid critical point in protein hydration water, which previously has been proposed only for bulk water [16].

Water molecules in a protein solution may be classified into three categories: (i) the bound internal water, (ii) the surface water, i.e. the water molecules that interact with the protein surface strongly, and (iii) the bulk water. The bound internal water molecules, which occupy internal cavities and deep clefts, are extensively involved in the protein-solvent H-bonding, and play a structural role in the folded protein itself. The surface water, or usually called the hydration water, is the first layer of water that interacts with the solvent-exposed protein atoms of different chemical character, feels the topology and roughness of the protein surface, and exhibits the slow dynamics. Finally, water, which is not in direct contact with the protein surface but continuously exchanges with the surface water, has properties approaching that of bulk water. In this letter, we deal with dynamics of the hydration water in a powder of the globular protein lysozyme. This hydration water is believed to have an important role in controlling the bio-functionality of the protein.

The biochemical activity of proteins also depends on the level of hydration. In lysozyme, enzymatic activity remains very low up to a hydration level \( h \) of \( \approx 0.2 \) (\( h \) is measured in g of water per g of dry protein) and then increases sharply with an increase in \( h \) from 0.2 to 0.5 [17]. Various experiments [18] and computer simulations [19]...
have demonstrated the strong influence of the hydration level on protein dynamics.

It has been found that many proteins exhibit a sharp slowing down of their functions (kinetic of biochemical reactions) at a temperature somewhere within the interval of $T$ between 200 K and 250 K. An analysis of the mean-squared atomic displacement, $\langle x^2 \rangle$, by using Mössbauer spectroscopy, X-ray, and neutron scattering spectroscopy, in hydrated proteins shows sharp changes around a certain sample temperature range: $\langle x^2 \rangle$ varies approximately linearly as a function of $T$ at low $T$ and then increases sharply above $T$ between 200 K and 250 K. The sharp rise in $\langle x^2 \rangle$ was attributed to a certain dynamic transition in biopolymers at this temperature range. The coincidence of the characteristic temperatures, below which the biochemical activities slow down, and the on-set of the dynamic transition, suggests a direct relation between these two phenomena. It has also been demonstrated that the dynamic transition can be suppressed in dry biopolymers, or in biopolymers dissolved in trehalose. It can also be shifted to higher temperatures, e.g. between 270 K and 280 K, for proteins dissolved in glycerol. Thus the solvent plays a crucial role in the dynamic transition in biopolymers. This observation led to a suggestion by many authors that proteins are ‘slaves’ to the solvent. Despite many experimental studies, the nature of the dynamic transition in proteins remains unclear. Many authors interpret the dynamic transition as a kind of glass transition in a biopolymer. Our experiments described below demonstrate that the origin of the characteristic temperature controlling both the activity of the protein and the transition in the behavior of $\langle x^2 \rangle$ is the FSC phenomenon in the hydration water, which shares the same crossover temperature with the protein.

Using high-resolution QENS method and the Relaxing-Cage Model (RCM, described in Methods section) for the analysis, we determine the temperature dependence of the average translational relaxation time, $\langle \tau_T \rangle$, for the hydration water. The dynamic crossover temperature of hydration water is defined as follows. At high temperatures, $\langle \tau_T \rangle$ follows a super-Arrhenius behavior (called a ‘fragile’ behavior) describable approximately by a Vogel-Fulcher-Tammann (VFT) law:

$$\langle \tau_T \rangle = \tau_1 \exp\left[\frac{DT_0}{(T - T_0)}\right],$$

where $D$ is a constant providing the measure of fragility and $T_0$, the ideal glass transition temperature at which the relaxation time appears to diverge. In reality, however, this divergence is avoided by the system. Instead, an Arrhenius behavior (called a ‘strong’ behavior) sets in below the crossover temperature $T_L$, where the functional dependence of the relaxation time switches to a law:

$$\langle \tau_T \rangle = \tau_1 \exp\left[\frac{E_A}{RT}\right].$$

In this equation, $E_A$ is the activation energy for the relaxation process and $k_B$, the Boltzmann constant. The crossover temperature $T_L$ is defined by the intersection of these two laws, which gives $1/T_L = 1/T_0 - (DK_B)/E_A$. In the case of hydration water in lysozyme, we found $T_L = 220$ K, which agrees well with the characteristic transition temperature in protein observed before. Since the average relaxation time $\langle \tau_T \rangle$ is a measure of the mobility of a typical hydration water molecule, this result implies that the sudden change in the trend of mobility of water molecules at the crossover temperature triggers the so-called glass transition of protein molecules.

We show in Fig. 1 as an example, a complete set (temperature series) of QENS area-normalized spectra taken at $Q = 0.87$ Å$^{-1}$ at ambient pressure. The broadening of the quasi-elastic peaks at the wing becomes more and more noticeable as temperature increases. And at the same time, the peak height decreases accordingly because the area is normalized to unity. In the inset C, we plot the peak height as a function of temperature. It is noticeable that the rate of increase as a function of temperature is different across the temperature 220 K. From Fig. 1B, we may notice, from wings of these spec-

![FIG. 1: Measured neutron spectra. Panels A and B show normalized QENS spectra at $Q = 0.87$ Å$^{-1}$, displaying the height of the peak (A) and the wing of the peak (B), respectively, at a series of temperatures. The inset C shows a plot of the peak heights versus temperature. Arrow signs in B are intended to highlight the wing of the spectra at the crossover temperature.](image-url)
average translational relaxation time, in an Arrhenius plot the temperature dependence of the mean-squared atomic displacement of the hydrogen atom calculated by Equs. (1-2) and at a series of temperatures. Panel B singles out one particular spectrum at $T = 230$ K and contrasts it with the resolution function of the instrument for this Q value (dash line).

In Fig. 3, we first present (in panel A) the mean-squared atomic displacement $\langle x^2 \rangle$ of the hydrogen atoms (calculated from the translational Debye-Waller factor, $S_H(Q, \omega = 0) = \exp[-Q^2\langle x^2 \rangle]$) versus $T$ to indicate that there is a hint of a dynamic transition at $T = 220$ K.

In particular at $T = 230$ K (panel B), we display the instrument resolution function purposely for comparison with the measured spectrum. RCM, as one can see, reproduces the experimental spectral line shapes of hydration water quite well. The broadening of the experimental data over the resolution function leaves enough dynamic information to be extracted by RCM. This means that it requires a high-resolution backscattering instrument with an energy resolution of 0.8 $\mu$eV to adequately study the FSC phenomena in hydration water.

In Fig. 3 we first present (in panel A) the mean-squared atomic displacement $\langle x^2 \rangle$ of the hydrogen atoms (calculated from the translational Debye-Waller factor, $S_H(Q, \omega = 0) = \exp[-Q^2\langle x^2 \rangle]$) versus $T$ to indicate that there is a hint of a dynamic transition at a temperature between 200 K and 220 K. This visual information obtained from the spectra before data analysis reinforces the result of the detail line shape analysis to be shown later in Fig. 4 that there is an abrupt dynamical transition at $T_L = 220$ K.

Recently, E. Mamontov observed a similar dynamic crossover in the surface water on cerium oxide powder sample [28]. The surface of cerium oxide is hydroxylated. The coverage of water is about 2 layers and the crossover temperature is said to be at 215 K. The observed slow dynamics is attributed to the effect on the translational mobility of the water molecules in contact with the surface of fragile liquid, quite closely. But at $T = 220$ K it suddenly switches to an Arrhenius law, a signature of a strong liquid. So we have a clear evidence of FSC in a cusp form. The $T_0$ for the fragile liquid turns out to be 176 K, and the activation energy $E_A = 3.13$ kcal/mol.
face hydroxyl groups. Thus, our observation of the FSC in hydration water of protein may be a universal phenomenon for surface water.

It should be noted that the FSC in confined super-cooled water is attributed to the crossing of the so-called Widom line in the Pressure-Temperature (phase) plane in a recent MD simulation work on bulk water \[12\] and protein hydration water at ambient pressure \[13\]. The Widom line is originated from the existence of the second critical point of water and is the extension of the liquid-liquid coexistence line into the one phase region. Therefore, our observation of the FSC at ambient pressure implies that there may be a liquid-liquid phase transition line in the protein hydration water at elevated pressures. This dynamic crossover, when crossing the Widom line, causes the layer of the water surrounding a protein to change from the ‘more fluid’ high-density liquid form (which induces the protein to adopt more flexible conformational sub-states) to the ‘less fluid’ low-density liquid structure (which induces the protein to adopt more rigid conformational sub-states).

In summary, an investigation of the average translational relaxation time, or the alpha-relaxation time, of protein hydration water as a function of temperature reveals a hitherto un-noticed Fragile-to-Strong dynamic crossover at 220 K, close to the universal dynamic transition temperature documented for proteins in literature. This fact implies that the sudden transition of the water mobility on the surface of a protein at the FSC triggers the so-called glass transition, which is known to have a profound consequence on biological function of the protein itself.

**Methods**

**Sample preparation**

Hen egg white lysozyme used in this experiment was obtained from Fluka (L7651, three times crystallized, dialysed and lyophilized) and used without further purification. The sample was dried under vacuum in the presence of P\(_2\)O\(_5\) to remove any water left. The dried protein powder was then hydrated isopiestically at 5 °C by exposing it to water vapor in a closed chamber until h = 0.3 is reached \(\text{i.e. } 0.3 \frac{g}{g} \text{H}_2\text{O per g dry lysozyme}\). The hydration level was determined by thermo-gravimetric analysis and also confirmed by directly measuring the weight of absorbed water. This hydration level was chosen to have almost a monolayer of water covering the protein surface \[20\]. A second sample was then prepared using D\(_2\)O in order to subtract out the incoherent signal from the protein hydrogen atoms. Both hydrated samples had the same water or heavy water/dry protein molar ratio. Differential scanning calorimetry (DSC) analysis was performed in order to detect the absence of any feature that could be associated with the presence of bulk-like water.

**Data Analysis**

QENS experiments essentially provide us with the Fourier transform of the Intermediate Scattering Function (ISF) of the hydrogen atoms, \(F_H(Q,t)\), of water molecules in the hydration layer. MD simulations have shown that the ISF of both bulk \[51\] and confined \[52\]...
supercooled water can be accurately described as a two-step relaxation: a short-time Gaussian-like (in-cage vibrational) relaxation followed by a plateau and then a long-time (time longer than 1 ps) stretched exponential relaxation of the cage. The RCM [24], which we use for data analysis, models closely this two-step relaxation and has been tested extensively against bulk and confined supercooled water through MD and experimental data [12, 31, 32]. By considering only the spectra with wave vector transfer $Q < 1.1 \text{ Å}^{-1}$, we can safely neglect the contribution from the rotational motion of water molecule in ISF [24]. The RCM describes the translational dynamics of water at supercooled temperature in terms of the product of two functions:

$$F_H (Q, t) \approx F_T (Q, t) = F^S (Q, t) \exp \left[ - (t/T_T (Q))^\beta \right] ,$$

$$T_T (Q) = \tau_0 (0.5Q)^{-\gamma}$$

where the first factor, $F^S (Q, t)$, represents the short-time vibrational dynamics of the water molecule in the cage. This function is fairly insensitive to temperature variation, and thus can be calculated from MD simulation. The second factor, the $\alpha$-relaxation term, contains the stretch exponent $\beta$, and the $Q$-dependent translational relaxation time $T_T (Q)$, which is a strong function of temperature. The latter quantity is further specified by two phenomenological parameters $\tau_0$ and $\gamma$, the exponent controlling the power-law $Q$-dependence of $T_T (Q)$. The average translational relaxation time, which is a $Q$-independent quantity, we use in this paper is defined as:

$$\langle \tau_T \rangle = \tau_0 \Gamma (1/\beta) / \beta ,$$

where $\Gamma$ is the gamma function. The temperature dependence of the three phenomenological parameters, $\tau_0$, $\beta$, and $\gamma$, are obtained by analyzing simultaneously a group of nine quasi-elastic peaks at different $Q$ values. Then the average translational relaxation time, $\langle \tau_T \rangle$, is calculated according to Equ. (2) using $\tau_0$ and $\beta$. As can be seen from Equs. (1) and (2), the product $\beta \gamma$ is an exponent expressing the $Q$-dependence of the ISF. Fig. 4 gives the temperature dependence of the exponent $\beta \gamma$, which indicates a precipitous drop at the crossover temperature 220 K from a high temperature value of about 1 to a low temperature value of about 0,2, while $\beta$ maintains a value of 0.5 all the way through 12. Note that the values of exponents $\beta$ and $\beta \gamma$ for free diffusion are 1 and 2, respectively, and the lower values of these two exponents signify an anomalous and more restrictive mobility of water compared to that of the free diffusion. The Fig. 4 shows that while the mobility of hydration water molecule at $ps$ to $ns$ timescale deviates significantly from free diffusion at all temperatures measured, as temperature goes below the crossover temperature $T_L$, the mobility becomes drastically reduced.

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