Some thermodynamical aspects of protein hydration water

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We study by means of nuclear magnetic resonance the self-diffusion of protein hydration water at different hydration levels across a large temperature range that includes the deeply supercooled regime. Starting with a single hydration shell ($h = 0.3$), we consider different hydrations up to $h = 0.65$. Our experimental evidence indicates that two phenomena play a significant role in the dynamics of protein hydration water: (i) the measured fragile-to-strong dynamic crossover temperature is unaffected by the hydration level and (ii) the first hydration shell remains liquid at all hydrations, even at the lowest temperature. © *2015 AIP Publishing LLC.* [http://dx.doi.org/10.1063/1.4921897]

An intriguing phenomenon is the way a complex system can progressively lose its freedom. In physics, the liquid-to-glass transition occurs as a function of such thermodynamic variables as temperature (T) and concentration (C),[1,2] and can be quantified by measuring the collective liquid structural and dynamic changes when T is decreased or C increased.[3,4] Despite the numerous efforts to identify the universal properties of the pre-vitreous supercooled liquid dynamic regime, many open questions remain.

Liquid water is a prototype supercooled liquid. Its well-known thermodynamic anomalies caused by its hydrogen-bond (HB) interactions determine its properties in both the stable and the supercooled phases. Liquid water is fundamental to life and is key in all living systems. The HBs in water drive the structure, dynamics, and functioning of biological macromolecules. In the life of cells and in protein-folding, water mediates the collapse of the chain and allows the onset of native topology through a funneled energy landscape.[5] Liquid H_2O in biology is thus not simply a solvent, but is itself a biocomponent, i.e., a "biomolecule," with a fundamental dynamic and structural role.[6]

Most researchers agree that the HBs between water molecules and the hydrophilic part of solutes are key to understanding the properties and functioning of water in biological environments. HB clustering causes such water anomalies as the density maximum and the diverging of various thermal response functions when the temperature is decreased into the supercooled region of the phase diagram.[7] As T is decreased, the HBs cluster form an open tetrahedrally coordinated HB network, experience an increase in both lifetime and cluster stability, and take on an altered local structure that can, in principle, continue down into the amorphous region of the phase diagram. Hence, liquid water is polymorphic, i.e., there is a "dynamic mixing" of molecules in the HB network of two liquids of differing densities, a low-density liquid (LDL) and a high-density liquid (HDL).[8]

Water can remain liquid from the melting temperature $T_M = 273$ K to the homogeneous nucleation temperature $T_H = 231$ K (the metastable supercooled phase). The glass transition temperature T_g is located in the $T < 130$ K region. Above T_g, water is a viscous fluid that crystallizes at $T_X \approx 150$ K. Between T_X and T_H in the bulk liquid water phase diagram, there is a "No-Man's Land" that is difficult to study experimentally.[2] Techniques for overcoming this difficulty include confining water in nanopores so small that the liquid cannot freeze[9] or spreading it over hydrate surfaces or larger molecules, such as biomolecules.[10] Studies using these techniques indicate that when T is decreased to a certain point, the water HB lifetime increases by many orders of magnitude. This in turn indicates that at $T_L \simeq 225$ K, there is a fragile-to-strong dynamic crossover (FSDC) and a violation of the Stokes-Einstein relation.[9,11,12] Below T_L, the LDL HB network structure predominates over the HDL structure.[10,13] The bulk water FSDC has been predicted to occur at $T_L \simeq 228$ K.[14]

The study of hydration water is key to understanding the essential processes that occur in proteins. Many studies using various techniques have been carried out on biological macromolecules[15–26] from the stable liquid phase to the supercooled regime. The reversible folding-unfolding process and the irreversible denaturation, that for lysozyme takes place at $T_D \approx 345$ K,[22–24,27] occur in the high-T regime, but to clarify our understanding of the function of hydration water in protein activity, an examination of the supercooled region is essential.[15,16,19,25,26]

Inside the No-Man's Land of the phase diagram, biomolecules undergo an important dynamic transition. When $T < 200$ K, dry proteins are in a solid-like structure—the protein "glassy" state—that has no conformational flexibility.[28] By increasing T the atomic motional amplitude, the mean-squared

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atomic displacement $\langle X^2 \rangle$ (MSD), increases linearly as in a harmonic solid. When the protein is hydrated, there is an onset of an additional anharmonic and liquid-like motion and the MSD sharply increases at ~220 K.[15,29–31] This transition is solvent dependent and thus does not occur in dry biomolecules. The biochemical activity of many proteins suddenly slows at temperature T_C in a universal interval 240–200 K and is strongly influenced by the hydration level h, i.e., the grams of H_2O per grams of dry protein.[32] The enzymatic activity in lysozyme is very low up to $h \sim 0.2$, but when h is increased from 0.2 to 0.5, the activity increases sharply. Note that the value $h = 0.3$ corresponds to a water monolayer covering the protein surface.[33]

Two types of water are present in hydrated proteins: bound internal water and hydration water. Hydration water is the first water layer and it strongly interacts with the protein surface. Bound internal water molecules are located in internal cavities and clefts, are key in the protein-solvent H-bonding process, and play a structural role in the folded protein itself. Hydration water interacts with the solvent-exposed protein atoms of a differing chemical character, follows the topology and roughness of the protein surface, and controls the biofunctionality of the protein.[34,35] Water can influence both the hydrophilic and hydrophobic side-groups of a biomolecule. The hydrophilicity (the HB strength) governs the secondary structure and folding specificity[36] of the biomolecule. Because the properties of the surface water (the first layer water network) strongly influence protein stability and function, understanding the hydrophilic interactions with the peptide groups is an important topic when studying biological systems.

Experimental evidence indicates that there is an approximate coincidence between the characteristic T_L temperature of the FSDC in water confined in nanopores and the T_C temperature for the slowing of biochemical activities. There is a $\langle X^2 \rangle$ sharp rise in hydrated biomolecules at the same temperature and this suggests a connection between the two phenomena. This protein dynamic transition is believed to be triggered by the strong HB coupling with the hydration water.[37] Note that the thermodynamic activity in water is characterized by a singular thermodynamically consistent temperature T^* for both the isothermal compressibility $K_T(T,P)$ and the coefficient of thermal expansion $\alpha_P(T,P)$. In particular, at $T^* \sim 315 \pm 5$ K, the $K_T(T)$ shows a minimum for all the studied pressures and all the $\alpha_P(T)$ curves measured at different P crossings.[38] Whereas T_D is the folding-unfolding temperature, T^* marks, by decreasing T, the water transition from a normal fluid to an anomalous, complex liquid.[9] In addition, as has been suggested by calorimetric and nuclear magnetic resonance (NMR) experiments, this temperature is the limit of the protein native state (when $T > T^*$, there is an onset of the unfolding mechanisms).[9,22]

Building on these observations and assuming the coupling between the protein and its hydration water, we carry out a nuclear magnetic resonance study on the self-diffusion $D_S(T)$ of hydration water across a very large T range, i.e., from the stable water liquid phase to the deeply supercooled regime. Our objective is to verify how the dynamic crossover in protein hydration water is dependent upon the hydration level. Using a variety of experimental methods,[30,31] e.g., neutron scattering (backscattering), broadband dielectric spectroscopy (BDS), and differential scanning calorimetry (DSC) on myoglobin (Mb) D_2O-hydrated powders ($0 < h < 0.5$), it has been found that myoglobin, like lysozyme, shows a FSDC that is caused by the water molecules strongly interacting with the protein surface at $T_L \sim 230$ K and that is largely unaffected by the hydration level. If we study water at the highest hydration levels where there is an excess of water in the first hydration shell that is subject to freezing,[39] we can determine whether this resultant ice affects the remaining liquid water in the first hydration shell.

We study the dynamic properties of lysozyme hydration water at different temperatures and at ambient pressure using a Bruker AVANCE NMR spectrometer operating at 700 MHz ^{1}H resonance. We measure the proton self-diffusion coefficient D_s using the pulsed field gradient stimulated technique (^{1}H-PFGSTE) and use a powder of the globular protein lysozyme hydrated at values in the interval $0.3 < h < 0.65$ according to a precise procedure.[19] We have to mention that, with the used experimental technique, only the water hydrogens contribute to the measured D_s. To be precise, the NMR technique in our experimental configuration is essentially sensitive only to translational motions and hence, the corresponding hydrogens of the protein side chain dynamics cannot be evaluated. The dried protein powder is hydrated at 5 °C by exposing it to water vapor in a closed chamber until the desired hydration level h is reached. We compare the results to $D_s(T)$ data at $h = 0.3$ and 0.32 previously studied in the temperature interval 200–360 K and carry out DSC to test for the absence of bulk-like water. In this case, the obtained results are coincident with previous experiments on the same system at about the same concentrations, and also for dry lysozyme.[40–42] This allows us to study the protein FSDC in the native protein state and the irreversible denaturation that takes place at $T_D \approx 345$ K,[9,22] and we can explore lysozyme hydrated at $h = 0.37, 0.48, 0.52, 0.61$, and 0.65 in the $200 < T < 293$ K range. The samples are cooled slowly and a T stability of 0.1 K is maintained during the experiments.

Figure 1 shows the measured proton self-diffusion of $D_s(T)$ data for the first series of the studied hydrations ($h = 0.37$, 0.48, and 0.61) as an Arrhenius plot (log D_s vs $1/T$). The bulk water data[43] (blue circles) and the corresponding data for $h = 0.3$ (red up triangles) and $h = 0.32$ (green down triangles) are also shown. Figure 1 also shows the two significant temperatures for water and proteins, T_D and T^*, together with the crossover temperature. Note (i) that the $D_s(T)$ temperature behavior of $h = 0.61$ for the jump at $T \sim 265$ K differs from that of the remaining hydrations; (ii) that all the other hydrations are similar to that in bulk water (they are super-Arrhenius), are a function of T in the region of moderate supercooling, can be described using the same scaling law $|T - T_L|^\gamma$ (that of the ideal mode-coupling-theory-MCT[4,44]), and the reported curves are the corresponding fits; (iii) that the measured T_L values for bulk water and for lysozyme at the different hydrations are, within the error bars, the same $T_L = 225 \pm 5$ K value (i.e., 228 K for the bulk water value,[14] 220 K for $h = 0.3$ and $h = 0.32$, 222 K for $h = 0.37$, and 224 K for $h = 0.48$); and (iv) that the γ values are 1.8 for bulk H_2O, 2.1 for $h = 0.3$ and $h = 0.32$, 2.2 for $h = 0.37$, and 2.15 for h

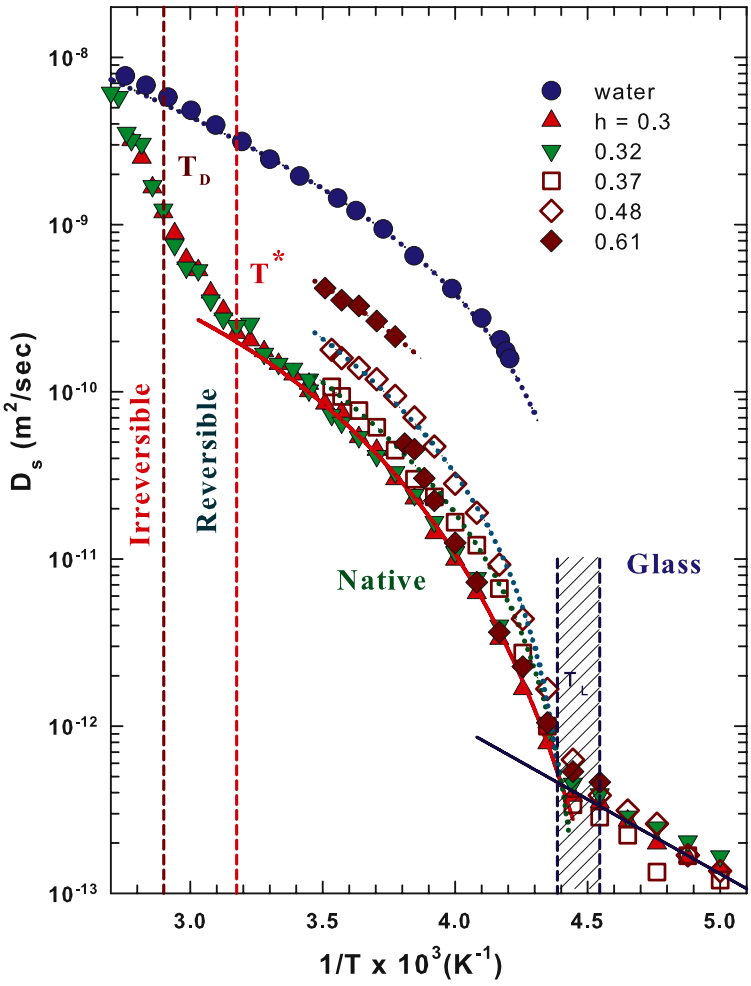

FIG. 1. The measured proton $D_s(T)$ data are reported in an Arrhenius plot (log D_s vs $1/T$). More precisely, data of pure bulk water[43] (blue circles) and the following hydrations: $h = 0.37$ (open squares), 0.48 (open diamonds), and 0.61 (full diamonds) are reported; for comparison, data from two previously studied hydrations $h = 0.3$ and 0.32 (red up triangles and green down triangles, respectively), corresponding nearly to a single hydration layer,[9,22] are also reported. The three protein significant temperatures are indicated: the crossover one T_L, T_D that identifies the folding-unfolding process and T^* that marks the water transition from a normal fluid to the anomalous and complex liquid, and the limit of the protein native state.[38] The reported curves are the data fits with the ideal mode-coupling-theory scaling law.[4,44] The shaded area indicates the interval in which fall the obtained MCT values for T_L. Finally, the $D_s(T)$ behavior at $h = 0.61$ shows a marked jump at $T \sim 265$ K.

= 0.48. Note that for $T > T^*$, a continuous evolution is evident for $D_s(T)$ because of the protein denaturation process at T_D. This is the case from values of the hydration water to those of pure bulk water, and this indicates a decoupling between the water and the protein.[9,22] In the figure, a shaded area indicates the interval in which fall the obtained MCT values for T_L. We must mention regarding the point (iii) that recent neutron scattering experiments are (within the experimental error) in agreement with our conclusions.[30,31,45]

Figure 1 thus shows that for samples of moderate hydrations ($h = 0.3$, 0.32, 0.37, and 0.48), T_L is independent of the protein hydration, but that for values obtained for γ, the observed protein hydration water dynamics are the result of HB interactions between the protein side-chains and the water.[9,22] If we take into account the extended MCT for activated hopping processes,[46,47] where T_L represents the MCT critical temperature below which the system dynamics are governed by extended clusters, we can explain the observed features of lysozyme hydration water. Figure 1 shows that samples with different hydration levels have a single hopping mechanism. This is indicated not only by the same crossover temperature but also by approximately the same Arrhenius activation energy for all hydration values. An estimation of this gives the value of 3.2 kcal/mol consistent with the values obtained, inside the same temperature interval for the same protein and myoglobin, measured by using neutron scattering.[19,48] A comparison with the corresponding dielectric relaxation (DR) times deserves a special comment. For many years, activation energies well different from those measured by NMR and neutrons have been proposed. However, accurate DR studies suggest for the measured spectra an additional weak and asymmetric relaxation ("fast" or "main" process,[49] process "1,"[50] and process "0"[31]) that if taken into account will give analogous thermal behaviors in the transport parameters measured by these spectroscopic methods in hydrated proteins (lysozyme and myoglobin). Such an analysis reflects the contribution to the observed DR spectra of all sample relaxing dipoles, especially for proteins where hydration water, polar protein side chains and backbone dipoles contribute.

A variety of experimental approaches to confined water in nanostructures and proteins (from Raman to neutron scattering including MD simulation studies[10–13,15,16,19,25,26,30,31,51,52]) have produced results that allow us to assume that the FSDC in water is caused by an evolution of the HDL to LDL, which supports the hypothesis that the "dynamic" transition in proteins is triggered by their strong HB coupling with the hydration water.

Note that in the T behavior of $h = 0.61$ below the jump temperature, the corresponding D_s follows the same behavior as the hydration water monolayer ($h = 0.3$ and $h = 0.32$). This suggests that in the lowest temperature regime where the

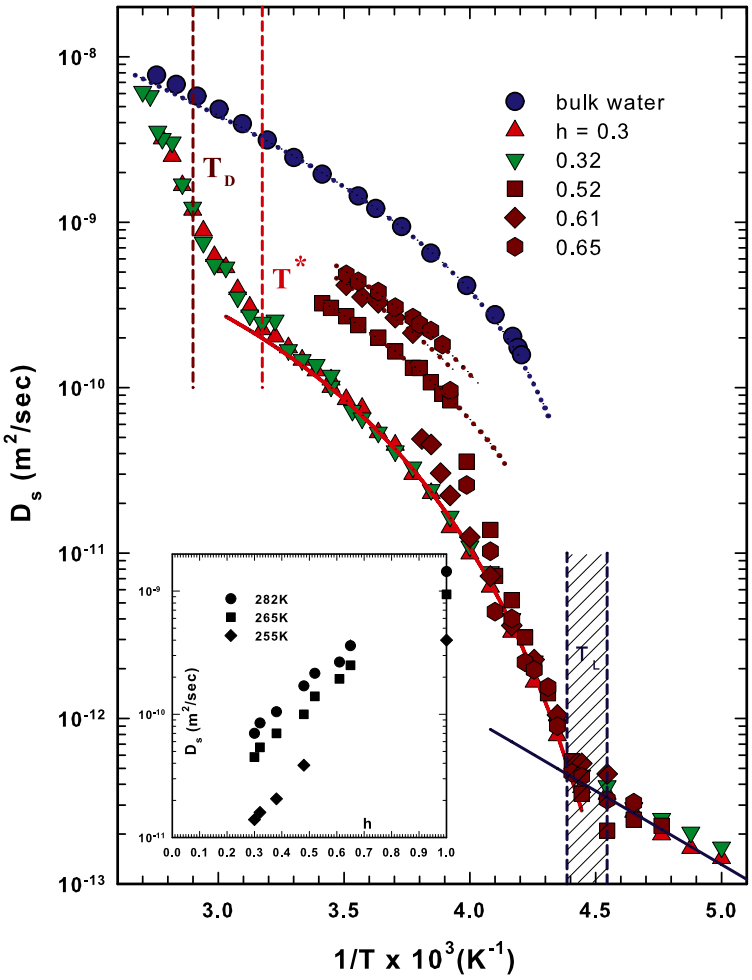

FIG. 2. The $D_s(T)$ data of pure bulk water, $h = 0.3$, 032, and 0.61 are shown, as in Figure 1. In addition, the proton self-diffusion data for $h = 0.52$ (full squares) and 0.65 (full hexagons), showing a similar behavior of 0.61, i.e., a jump to the $D_s(T)$ values of a water monolayer around the protein surface, are displayed. The inset illustrates the hydration dependence of D_s in the temperature region where all the protein hydration water remains liquid.

"excess" protein hydration water freezes, the first hydration shell remains liquid. Experimentally, the NMR setup used detects liquid water only, i.e., ice is outside the instrument's resolution. When the liquid water protons are present at the experimental temperature, they are detected and contribute to the NMR spectra, but ice protons only contribute to the spectral background. This confirms the findings of calorimetric studies on metmyoglobin, methemoglobin, and lysozyme proteins that in water at the highest hydrations, where there is an excess of water in the first hydration shell, that excess water can freeze without affecting the liquid water remaining in the first hydration shell.[39] Figure 2 shows that these results are confirmed in experiments with hydrations $h = 0.52$ and $h = 0.65$. Here, the dotted lines represent the MCT fits with analogous results, with respect to the previously studied hydrations, on the exponent γ and the crossover temperature T_L. Again, the influence of the HBs on the properties of protein hydration water is confirmed. On the basis of these results, we consider that new DSC experiments can fully illustrate the freezing of the water in excess and at the same time can clarify this phenomenon on more appropriate quantitative terms. We plan to use for this purpose the modulated calorimetry technique.[42]

The hydration dependence of the self-diffusion coefficient $D_s(h)$ in both the normal and supercooled temperature regions indicates that all the protein hydration water remains liquid. The inset of Fig. 2 shows the $D_s(h)$ behavior at fixed temperatures $T = 255$ K, 265 K, and 282 K on a log-linear scale. Note that the evolution is approximately temperature-independent, which indicates that the dynamics of bulk and protein hydration water are closely similar and supports the hypothesis that the physical properties of hydrated proteins are due to the coupling of the hydrophilic side chains with the dynamics of the hydration water.

In conclusion, our experiment indicates that two phenomena dominate the dynamics of protein hydration water: (i) the crossover temperature is unaffected by the hydration level, and it is approximately the same as that predicted for bulk water[14] and that measured in water confined inside nanopores,[9–12] and (ii) the first hydration shell remains liquid at all hydrations. Both of these results are significant. The first supplies further proof that water triggers the "dynamic" protein transition.[10–13,15,16,19,25,26,30,31,51,52] The second explains why proteins (in their dynamic and biological properties) survive inside the No-Man's Land. The data indicate that the first hydration layer plays a bioprotective role by retaining liquid at low temperatures. It protects the functioning of the local protein conformational dynamics and disallows irreversible changes in the molecule's structure.

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