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Water and lysozyme: Some results from the bending and stretching vibrational modes

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The dynamic or glass transition in biomolecules is important to their functioning. Also essential is the transition between the protein native state and the unfolding process. To better understand these transitions, we use Fourier transform infrared spectroscopy to study the vibrational bending and stretching modes of hydrated lysozymes across a wide temperature range. We find that these transitions are triggered by the strong hydrogen bond coupling between the protein and hydration water. More precisely, we demonstrate that in both cases the water properties dominate the evolution of the system. We find that two characteristic temperatures are relevant: in the supercooled regime of confined water, the fragile-to-strong dynamic transition occurs at T_L , and in the stable liquid phase, $T^* \simeq 315 \pm 5$ K characterizes the behavior of both isothermal compressibility $K_T(T, P)$ and the coefficient of thermal expansion $a_P(T, P)$.

Keywords protein unfolding, hydration water, infrared spectroscopy

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1 Introduction

Although water is a simple molecule, it has intriguing and counterintuitive physicochemical behaviors that have not been clearly explained [1]. The enormous number of studies on water have all come to the same conclusion: hydrogen bond (HB) interactions between water molecules are the key to understanding its properties and functioning [2], especially in biological environments [3]. As the temperature T decreases, the HBs cluster and form an open tetrahedrally coordinated HB network. This HB clustering explains such thermodynamic anomalies as the density maximum at 4°C and the diverging behavior of various thermal response functions when the temperature is decreased into the supercooled region of the phase diagram. More precisely, when the temperature of the stable liquid phase is lowered, both

the HB lifetime and cluster stability increase, and this altered local structure can, in principle, continue down to the amorphous region of the phase diagram.

Water is not a simple solvent in biosystems. It controls their stability, dynamics, and function [4] and takes an active role in, e.g., the protein folding–unfolding process and the life of cells [5]. Understanding the contribution of HBs to protein stability and to the structural flexibility and dynamics of macromolecules enables us to understand the complex properties of water and their influence on the behavior of biological systems.

Below $T_g \approx 130$ K, water is a glass. Above that temperature, it becomes a highly viscous fluid that crystallizes at $T_X \approx 150$ K. Amorphous water, like ice, is polymorphic. The two phases of glassy amorphous water, low density (LDA) and high density (HDA), can be transformed from one to the other by tuning the pressure. Metastable supercooled water is located on

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the phase diagram between the homogeneous nucleation temperature, $T_H = 231$ K, and the melting temperature, $T_M = 273$ K. The region between T_X and T_H is not experimentally accessible for bulk liquid water and is referred to as the “no man’s land” of the phase diagram, but crystallization within this region can be retarded somewhat by confining the water within narrow nanoporous structures or on a protein surface.

The polymorphism of glassy water and the HB networking suggest that liquid water may also be polymorphous, i.e., a mixture of a low-density liquid (LDL) and a high-density liquid (HDL) that predominates in the high- T regime. In the HDL phase, the local tetrahedrally coordinated HB structure is not fully developed, but in the LDL, a more open ice-like HB network appears. Thus, water’s anomalies are caused by the competition between these two local liquid forms. Studies of confined water [6, 7] have found that decreasing T greatly increases the water HB lifetime, indicating the presence of a dynamic crossover from a super-Arrhenius (fragile) to an Arrhenius (strong) liquid at $T_L \simeq 225$ K and ambient pressure. At this temperature, the Stokes–Einstein relation is violated [6, 8], and clear signs of LDL and HDL are observed [7], suggesting that polymorphism also exists in the liquid phase.

This fragile-to-strong dynamic crossover (FSDC), with $T_L > T_g$, is not limited to water but also characterizes all supercooled glass-forming liquids [9]. Its appearance indicates that the system is approaching dynamic arrest [9–17], and many special processes occur at that point (see, e.g., Ref. [12]), including violation of the Stokes–Einstein law, orientational–translational decoupling [18], splitting of the relaxation into primary (α) and secondary relaxation times (β), the onset of dynamical heterogeneities, and the onset of the boson peak (hypothesized for bulk water [15] and observed in confined water [16]).

The FSDC is highly relevant in biology because it is related to the dynamic transition that biomolecules undergo in the low- T regime [17, 19–23]. At the lowest temperatures, proteins are in a glassy state, a solid-like structure without conformational flexibility [24]. When T is increased, measurements of the mean-squared atomic displacement (MSD), $\langle X^2 \rangle$, reveal that the atomic motional amplitude increases linearly, as in a harmonic solid. In hydrated proteins, its rate suddenly increases at ~ 220 K, signaling the onset of an additional anharmonic and liquid-like motion [25, 26]. The functions and kinetics of the biochemical reactions in many proteins slow down sharply at a temperature T_C in a universal range of 240–200 K [27]. This transition can be suppressed in dry biomolecules [26], and the protein biochemical activity depends on their level of hydration h (i.e., grams of

H₂O per grams of dry protein). For lysozyme, $h = 0.3$ corresponds to a water monolayer covering the protein surface; the enzymatic activity is very low up to a hydration level of 0.2 and then increases sharply as h increases from 0.2 to 0.5 [28].

In a protein–water solution, water can influence both the hydrophilic and hydrophobic side groups of a biomolecule, and in this respect two water categories are of interest: bound internal water and surface (or hydration) water. The first layer of water, in strong interaction with the protein surface, is the hydration water. The bound internal water molecules, located in internal cavities and clefts, are extensively involved in protein–solvent H bonding and play a structural role in the folded protein itself. The hydration water, interacting with the solvent-exposed protein atoms having different chemical character, feels the topology and roughness of the protein surface and is believed to have an important role in controlling the biofunctionality of the protein [29, 30].

The study of the physical properties of confined water depending on the cavity surface and/or the confinement dimensions is a very active field. Indeed, water structures and dynamics in confined environments are studied to improve knowledge of geological and biological processes. However, despite the numerous experimental and theoretical studies, the behavior of water at hydrophilic and hydrophobic interfaces is still not completely understood [31, 32].

Hydrophilicity (which results from the HB strength) is a force that governs the secondary structure and folding specificity of proteins [33], but the properties of the biomolecule can also be affected by the protein methyl groups. It has been shown that although a single hydration water layer can influence both the hydrophilic chains and the dynamics of the entire protein, the dynamic effects on the methyl chains are minimal (their motions are confined and attributed to librational and rotational movements). Several hydration shells of water are required for the hydrophobic side chains to exhibit, at room temperature, the full range of motions characteristic of a liquid-like protein state [34]. Hence, because the properties of the hydration water are intimately connected to protein stability and function, an understanding of the hydrophilic interactions with peptide groups is essential for the study of biological systems.

The approximate coincidence of T_L (the water dynamic crossover temperature) and T_C (at which biochemical activities slow and $\langle X^2 \rangle$ rises sharply in biomolecules) has suggested a connection between the two phenomena. Specifically, this dynamic transition in proteins is believed to be triggered by their strong HB coupling with the hydration water [35]. This behavior has

been observed in several biomolecules, including globular proteins [19, 20], DNA, and RNA [21].

This dynamic crossover in protein water has been studied by measuring the MSD and the transport parameters — the self-diffusion coefficient D_S and the average translational relaxation time $\langle\tau\rangle$ as a function of T — using various experimental techniques ranging from neutron scattering [19] to nuclear magnetic resonance (NMR) [20].

The FSDC can be observed in water only under confinement, but there is another transition that characterizes thermodynamically stable bulk water. Recent studies of water density as a function of both temperature and pressure [36–38] propose the existence of a “special” temperature, $T^* \simeq 315 \pm 5$ K, that characterizes the behavior of both the isothermal compressibility $K_T(T, P)$ and the coefficient of thermal expansion $\alpha_P(T, P)$ [39, 40]. More precisely, $K_T(T, P)$ shows a minimum for all the studied pressures at T^* , and all the $\alpha_P(T) = 1/V(\partial V/\partial T)_P$ curves, measured at different pressures P , cross at a “singular and universal expansivity point” located at exactly the same temperature, T^* ($\alpha_P(T^*) \sim 0.44 \times 10^{-3} \text{ K}^{-1}$). This temperature marks the curvature of $\alpha_P(T)$. Unlike other water singularities, this temperature is thermodynamically consistent with the relationship connecting the two response functions. Analysis of transport parameters such as the viscosity and the self-diffusion coefficient D_S has revealed that decreasing the temperature to T^* causes decoupling of the roto-translational dynamics [41] and a crossover from strong to fragile dynamics to occur (the opposite of what happens at T_L) [40]. The P – T behavior of the self-diffusion coefficient D_S and of the NMR proton chemical shift δ indicates that if we decrease T , at T^* a crossover from the normal fluid to the anomalous fluid occurs, and we have a complex liquid characterized by many anomalies.

In this work, we study the Fourier transform infrared (FTIR) spectra to verify how these two temperatures, T_L and T^* , which characterize confined water in the supercooled state and bulk water in the stable phase, respectively, can influence protein dynamics.

FTIR spectroscopy is among the experimental techniques most suitable for studying the structure and dynamics of proteins in aqueous solutions [42]. In fact, the different amide bands found in IR spectra (see the Results and Discussion) provide information about the secondary structure of proteins, which depends on the protein configuration. Their study, together with that of the water bands, can shed light on the mechanisms that govern the system’s thermodynamic properties.

2 Experiments

We study the globular protein lysozyme hydrated with a single monolayer of water. Samples are prepared by hydrating dried protein powder isopiesticly at 5°C by exposing it to water vapor in a closed chamber until the desired hydration level h is reached (here we use $h = 0.3$ and 0.37). We use differential scanning calorimetry to test for the absence of bulk-like water.

We take FTIR absorption measurements at ambient pressure using a PerkinElmer Spectrum GX Fourier transform spectrometer with an attenuated total reflection geometry. We record the measured spectra at a resolution of 4 cm^{-1} by performing 250 repetitive scans in order to obtain a high reproducibility and then normalize them. We initially cool the samples to 180 K and then measure them while increasing T in 10 K steps to 350 K, i.e., from below the protein dynamic crossover temperature in the protein native state to above the irreversible denaturation temperature ($T_D \approx 345$ K). The temperature increase is slow, and its stability is maintained in a range of 0.1 K.

3 Results and discussion

Figure 1 shows the vibrational bending mode (measured at 300 K) of pure bulk water (upper panel) and the peptide modes (amide I, amide II) falling in the spectral region $1300 \text{ cm}^{-1} < \nu < 1800 \text{ cm}^{-1}$ (lower panel). The peptide groups are described using nine characteristics bands in the IR and Raman spectra; the bands are designated amide A, B, and I–VII in order of decreasing frequency. Amides I and II are the two major bands of the protein IR spectrum. Amides III and IV, resulting from a mixture of several coordinate displacements, are very complex. Out-of-plane motions are found in amides V, VI, and VII. Amides I, II, and III are used to assign the protein secondary structure [44] and fall within the frequency range in which the water bending modes are located (1300 – 1800 cm^{-1}). Water has two bending modes, one caused by molecules clustered in the network (i.e., only the four-bonded or LDL, at $\sim 1560 \text{ cm}^{-1}$), and a second due to the remaining, nonclustered free network molecules (the HDL, at $\sim 1640 \text{ cm}^{-1}$) [43]. Amide I, which is determined by the peptide backbone conformation (and is independent of the amino acid sequence, its hydrophilic or hydrophobic properties, and its charge), contributes (i) the α helix (1650 – 1657 cm^{-1}), (ii) the antiparallel β sheets (two contributions in the ranges 1612 – 1640 cm^{-1} and 1680 – 1689 cm^{-1}), and (iii) the random

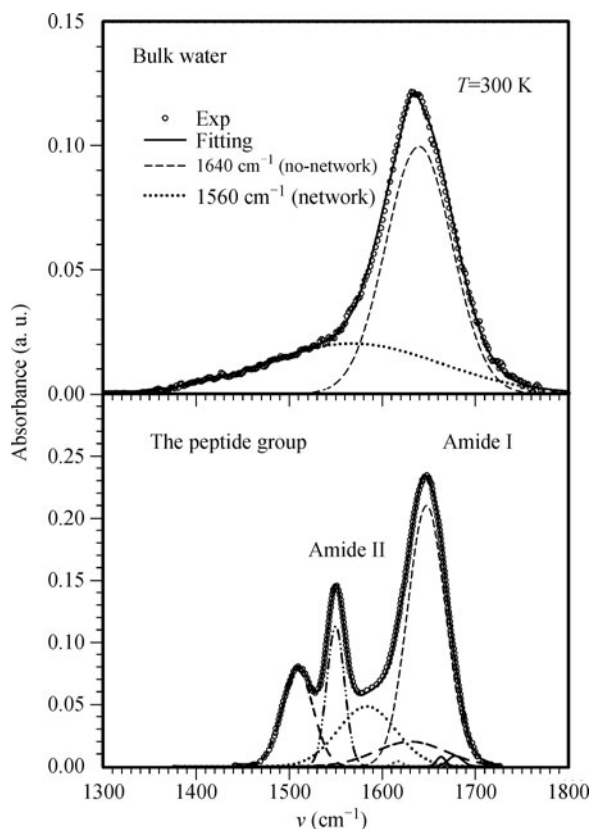


Fig. 1 The bending spectrum of pure bulk water at 300 K (top) in the range $1300\text{ cm}^{-1} < \nu < 1800\text{ cm}^{-1}$. The peptide groups, Amide I, Amide II, falling in the same spectral range (bottom panel). Reproduced from Ref. [5].

coil near 1680 cm^{-1} . The denatured proteins in the native state exhibit highly differentiated spectra [45]. There is a process characterizing the unfolding in the amide II band, and the amide II $N-H$ residual disappears ($1530\text{--}1550\text{ cm}^{-1}$).

Amide I absorption is caused by $C=O$ stretching vibration, and amide II absorption is due to $N-H$ bending vibration and $C-N$ stretching vibration at 1540 cm^{-1} and 1520 cm^{-1} , respectively. Using this information, a study of the bending vibrational mode of water and protein reveals that the HBs in water molecules with a protein peptide carbonyl oxygen ($C=O$) and an amide ($N-H$) molecular group trigger the biomolecular dynamic transition [5, 46, 47]. The most stable configuration has two HBs, a water proton donor bond to the carbonyl oxygen and an amide $N-H$ proton donor bond to the water oxygen [46, 47]. Water acts as an HB “glue” between the protein’s carbonylic and amidic groups [48] by governing the protein folding and secondary structure. All of this affects both the frequency and the extinction coefficient of IR absorption lines because the normal mode force constant is modified.

Figure 2 shows the vibrational OH stretching spectra

($2700\text{ cm}^{-1} < \nu < 3700\text{ cm}^{-1}$) of the water-lysozyme system with a hydration level of $h = 0.3$ at several different temperatures in the range $180\text{ K} < T < 350\text{ K}$. For comparison, it also shows the corresponding spectrum of pure bulk water measured at 293 K. Note that there is a marked difference between the bulk water data (blue circles) and hydrated protein data (blue line) at the same temperature. There is a large red-shift in the central O-H stretching frequency of protein hydration water ($\sim 50\text{ cm}^{-1}$) relative to the bulk. The shift increases toward lower temperatures and is characterized by a marked increase in the intensity of the hydration water in the region of the lowest frequencies, which is consistent with the contraction and increasing strength of the protein-water bonds; i.e., there is also a marked increase in the population of the bonded water. Unlike bulk water molecules, most water hydration molecules are bonded (with the protein hydrophilic group or in a water-water HB) [2]. Note that the spectra in Figs. 1 and 2 (where the protein-water vibrational dynamics change significantly with T) differ completely from those of crystalline and amorphous ice, indicating that the water is in a liquid phase.

The study of water and water solutions has traditionally focused on molecular stretching modes that are easily accessible using standard Raman and IR spectroscopic techniques because of their high absorption cross sections and frequencies. The relaxation of vibrational excitations in water reflects its physical properties [44]. The bending modes also exhibit analogous features because they are sensitive to the HB frequency, HB strength, and water molecule connectivity.

Bending mode relaxation differs from stretching be-

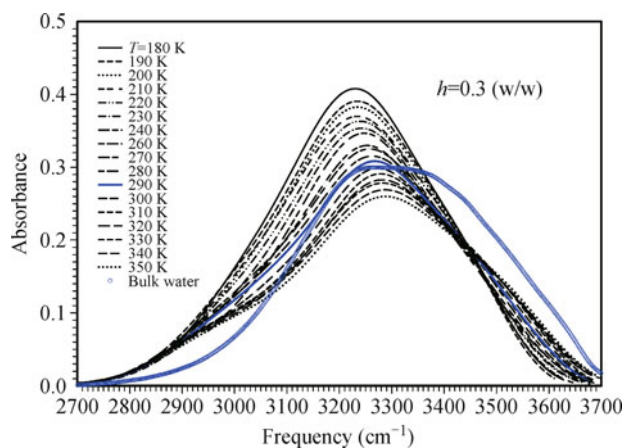


Fig. 2 The measured FTIR vibrational OH-stretching spectra ($2700\text{ cm}^{-1} < \nu < 3700\text{ cm}^{-1}$) of the water-lysozyme system with a hydration level of $h = 0.3$, at several different temperatures in the range $180\text{ K} < T < 350\text{ K}$; for comparison the corresponding spectrum of the pure bulk water measured at 293 K is also reported.

cause it occurs through intermolecular interactions and consequent energy transfers (stretching is essentially an intramolecular mode). These couplings occur in pure systems and in solutions, with the difference that in solutions these intermolecular interactions involve an energy transfer between solute and solvent (i.e., protein and water) [51].

Figure 1 shows the water contribution to the amide 1 bending mode. Walrafen et al. [44] use this spectral component to determine the spectral contributions of water inside (LDL) and outside (HDL) the network; the corresponding data can be used to evaluate the enthalpy value H of the HB formation process. For this, the van't Hoff expression, which relates the T dependence of an equilibrium constant K to the change in H as $d \ln K/d(1/T) = -\Delta H/R$, can be used. We extracted these two contributions using a spectral deconvolution with Gaussian functions [42, 44]. Figure 3 shows a logarithmic plot of the ratio A_{NET}/A_{NC} of the integrated areas of the fully tetrabonded molecules (1560 cm^{-1}) and the molecules outside the network and not clustered (1640 cm^{-1}) versus $1/T$. To clarify, we assume that the ratio A_{NET}/A_{NC} is directly related to K , and, from $\ln(A_{NET}/A_{NC}) = -\Delta H/RT$, we obtain the HB enthalpy variation in the large T interval under study. We examine data for bulk water (from experiments and from Ref. [44]) and confined water (in MCM nanotubes [50] and lysozyme at $h = 0.3$ and 0.37).

Figure 3 shows the results and confirms that there are different regimes immediately above and below the main temperatures characterizing the HBs, T_L and T^* , of the system. When we decrease T below the high-temperature regime of stable bulk water, the ratio first shows a flat minimum near T^* and then increases linearly to the

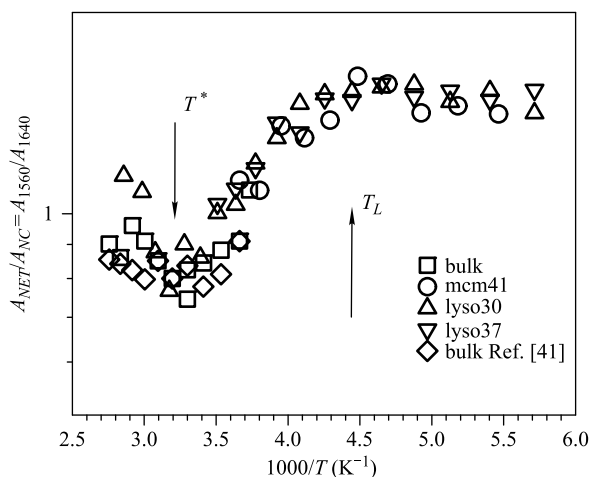


Fig. 3 The ratio A_{NET}/A_{NC} on a logarithmic scale versus $1000/T$ ($180 \text{ K} < T < 365 \text{ K}$). Data of bulk (experiments and Ref. [44]) and confined water (in MCM nanotubes [50] and lysozyme at $h = 0.3$ and 0.37) are reported.

FSDC temperature (T_L). Below the crossover temperature, it remains constant. This confirms the physical roles of T^* and T_L . At T^* , a tetrabonded water cluster is created, and at the FSDC (the onset of the protein harmonic solid-like state), a majority of the water molecules are linked to either the tetrabonded water cluster (the network or LDL phase) or to the protein by $C = O - HO$ and $NH - O$ bonds. The probability that an HB will form or break is low, and the only water motion is caused by hopping between clusters. Increasing T allows HBs in the anharmonic protein liquid phase to be formed and broken, and the corresponding probability evolves with T according to the van't Hoff equation. The enthalpy change ΔH of the water HB can be evaluated (i.e., $\sim 2.3 \text{ kcal/mol}$, typical of HBs) by plotting between these two main temperatures.

Finally, we quantitatively analyze the red-shift in the central frequency of OH stretching of protein hydration water (see Fig. 2), which may be the molecular mechanism underlying two dynamic transitions: (i) the transition originally observed in neutron scattering of the MSD of the hydrated protein, indicating the onset of an additional anharmonic liquid-like motion [25, 26] strongly related to the dynamical crossover, and (ii) the transition at the onset of the stable tetrahedral HB structure that characterizes water and its thermodynamic anomalies. Figure 4 shows the OH stretching frequencies of hydrated lysozyme at hydration levels of $h = 0.3$ and 0.37 as a function of the temperature in the interval $180 \text{ K} < T < 365 \text{ K}$. We increase the temperature and observe three linear behaviors: one for $T < T_L$, one in the interval between T_L and T^* , and one for $T > T^*$. These behaviors highlight the importance of both T_L and T^* and indicate how, because of the HBs between the water and the water-protein molecules, the dynamics of water vibration change the structure of the hydration water. This is caused by the correlation between the temperature dependence of the O-H stretching frequency and

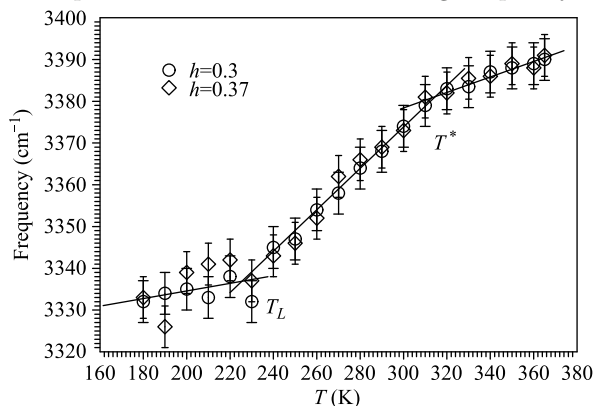


Fig. 4 The frequency behaviors of the protein water OH stretching contribution illustrated as a function of the temperature T .

the thermal expansion characteristic of hydrogen-bonded systems [52].

In structured (i.e., quasi-harmonic solid or solid-like) systems, the thermal expansion $\alpha_P(T)$ is linked to the normal mode frequencies $\nu(n)$ via the so-called Grüneisen parameters of mode $\gamma(n)$. In particular, $\nu(n)$ decreases as the volume increases ($\nu(n) \propto V^{-\gamma(n)}$). Thus, the relative changes of the mode frequencies with T are determined by the volume expansion coefficient ($-\partial\nu(n)/\partial T \sim (\gamma(n)\nu(n))\alpha(T)$). Because a quantitative correlation between the OH stretching frequency and the O–O separation has been observed in ice I [52], we assume that it holds also for water in tetrabonded structures and LDL clusters. The force constants in this structured water depend on the strength of the HB, which affects the OH stretching vibration. Thus, the OH stretching frequency increases with T , and the thermal expansion (the increase in the lattice parameters) reflects the behavior of a “linear Grüneisen relation” [52, 53] and generates new HB configurations (i.e., changes the structure). Figure 2 shows this behavior above and below T_L , where the structural change in confined water is accompanied by the protein dynamic transition. Although this clarifies T^* and shows it to be the temperature at which the curvature of bulk water $\alpha_P(T)$ changes, the frequency change around T^* is even more interesting [39, 40]. Recent NMR experiments focusing on the protein hydrophilic (amide NH) group, the hydrophobic (methyl CH₃) group, and methine (CH) indicate that T^* may represent the high-temperature limit of the protein native state [54].

4 Conclusion

In conclusion, our findings focus on how HB networking in water affects protein hydrophilic groups. The hydrophobic groups that appear when the protein is in its native folded state (i.e., up to T^*) are buried within the protein interior [4]. In the wide temperature region spanning the protein glass state to the onset of unfolding, the interaction between water and the carbonylic and amidic groups of the protein plays an important role together with the HB network of the hydration water [48]. This is the basis of protein folding and its secondary structure, and also of the two crossovers observed in the bending and stretching modes of the hydrated lysozyme. Below T^* , water cross-links with the protein hydrophilic groups, and above T^* , the HBs become weaker and allow the protein to unfold [38, 40].

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