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RESEARCH ARTICLE

Dynamical changes in hydration water accompanying lysozyme thermal denaturation

Francesco Mallamace[1,2,3,†], Carmelo Corsaro[1,2], Domenico Mallamace[4], Nicola Cicero[4], Sebastiano Vasi[2], Giacomo Dugo[4], H. Eugene Stanley[3]

[1] *CNR-IPCF Messina, Istituto per i Processi Chimico-Fisici, Viale F. Stagno D'Alcontres 37, 98158 Messina, Italy*
[2] *Dipartimento di Fisica e Scienze della Terra, Università di Messina, Viale F. Stagno D'Alcontres 31, 98166 Messina, Italy*
[3] *Center for Polymer Studies and Department of Physics, Boston University, Boston, MA 02215, USA*
[4] *Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti, e della Salute, Università di Messina Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy*
Corresponding author. E-mail: †francesco.mallamace@unime.it
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We study the dynamics of the first hydration shell of lysozyme to determine the role of hydration water that accompanies lysozyme thermal denaturation. We use nuclear magnetic resonance spectroscopy to investigate both the translational and rotational contributions. Data on proton self-diffusion and reorientational correlation time indicate that the kinetics of the lysozyme folding/unfolding process is controlled by the dynamics of the water molecules in the first hydration shell. When the hydration water dynamics change, because of the weakening of the hydrogen bond network, the three-dimensional structure of the lysozyme is lost and denaturation is triggered. Our data indicates that at temperatures above approximately 315 K, water behaves as a simple liquid and is no longer a good solvent.

Keywords lysozyme unfolding, hydration water, NMR, correlation time, solvent dynamics

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1 Introduction

Proteins in their unfolded state are a linear chain of amino acids without a stable three-dimensional structure [1, 2]. Under the right thermodynamic conditions, such as temperature and hydration, amino acids follow a precise physical route and rapidly evolve into a characteristic protein structure — a native protein state [3]. The bio-functionality of a protein is directly linked to the amino acid sequence and structure that characterizes the native protein state; furthermore, it is strongly dependent on the solvent, i.e., water, which actually functions as an amino acid [4]. Dry proteins exhibit no biological activity and require at least a water monolayer (a first hydration shell) covering their surface to function as proteins [5–7]. Water in the form of internal water is also an essential part of the three-dimensional structure of a protein [8, 9].

Many studies have probed the thermal limit of the biological activity of protein and have linked its onset at low temperatures to the occurrence of a dynamical crossover in the hydration water at approximately 225 K [10–20]. Although this precise value of temperature has been debated, given the possibility of inadequate instrumental resolutions, there is a consensus that the crossover occurs and that it is important [21–31].

The hydrophobic interaction is an important driving force in the aggregation and folding processes in proteins [32], in particular, in the burial effect of amino acid residues in protein interiors [33]. However, the process that maintains their stability and functionality is the hydrophilic interaction [27, 34, 35]. The hydrogen bonds (HB) formed between hydrophilic protein groups and water molecules are vital to protein life [4]. In addition, the thermodynamic properties of water, governed by the lifetime and stability of the HBs, influence the protein activity [27, 28, 36] to the extent that the slaving concept and model is used to describe how solvent fluctuations determine protein dynamics and functions [37, 38]. For example, the temperature above which water is no longer a good solvent and loses its anomalous

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fundamental properties is approximately 315 K. Infact, the lifetime and stability of the HBs dramatically decrease with increasing temperature and above $T^* \simeq 315$ K water behaves as a simple liquid [39].

The folding/unfolding process of proteins is controlled by variables such as pH, temperature, solvent, and time [6, 40, 41]. Recent studies on the hydrated lysozyme have determined the characteristic temperatures of the process. Although the protein begins to lose its three-dimensional structure above 315 K, if the temperature of the system can be maintained below $T_D = 346$ K for a short period of time, the unfolding mechanism can be stopped and reversed [42, 43].

The thermal denaturation of the lysozyme has been studied using such techniques as calorimetry, nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy [13, 14, 43, 44]. The process is kinetic and moves through three states: the native state $\leftrightarrow$ the reversible unfolding state $\rightarrow$ the irreversible denatured state. The first is initiated at ~ 315 K and can be reversed under certain conditions and the second is initiated at ~ 346 K and cannot be reversed.

The dynamics of the first hydration shell of water surrounding the protein surface is slower than the dynamics of the bulk solvent [45–47]. This is due to the complex network of hydrogen bonds that are formed between the water molecules and protein residues [6, 48, 49]. By increasing the temperature from the stable condition towards denaturation, however, the dynamics of hydration water change dramatically and trigger the unfolding process.

We use nuclear magnetic resonance to study the translational and rotational contributions made by the dynamics of lysozyme hydration water. The goal is to understand the dynamical changes that are responsible in driving protein denaturation.

In particular, we measure the translational contribution to the dynamics by studying the self-diffusion coefficient measured for hydrated lysozyme with a single hydration shell. In addition, we measure the spin-lattice relaxation time and extract the reorientational correlation time. We compare these parameters, evaluated for the hydration water, with the corresponding parameters for bulk water and find a strict connection between the dynamic water properties and the activity of a protein moving toward denaturation.

2 Experiments

To measure how the dynamics of the first hydration shell of water molecules surrounding the protein surface change, and to link it with the protein thermal limits of functioning (the unfolding process), we examined a protein lysozyme (a small protein of 14.4 kDa) with a hydration level (i.e., grams of water per gram of dry protein) of $h = 0.3$. We purchased lysozyme samples from Fluka (L7651 three times crystallized, dialyzed, and lyophilized), dried them, and then hydrated them isopiestically [13].

We used NMR (a 700-MHz Bruker Avance spectrometer) at one atmosphere to measure the proton self-diffusion coefficient of lysozyme hydration water [14, 28]. We performed the measurements using the pulsed field gradient stimulated echo (PGSTE) technique [50] with a field gradient value of 1200 Gauss/cm [14].

We also measured the proton spin-lattice relaxation time, T_1, focusing on the thermal region of the denaturation, 280 K $< T <$ 360 K [14, 29]. We used the inversion-recovery pulse sequence [51] to obtain the T_1 data and varied the interpulse delay from microseconds to several seconds. The data fit a weighted double exponential form,

$$\frac{M}{M_0} = 1 - 2\left[P\exp\left(-\frac{t}{T_{1s}}\right) + (1-P)\exp\left(-\frac{t}{T_{1f}}\right)\right], \tag{1}$$

where P is the weight, t the interpulse delay variable, and T_{1s} and T_{1f} are the two relaxing contributions. Figure 1 shows the curve fitting for two different temperatures.

The slowest relaxing component T_{1s} (in the order of seconds) belongs to water protons in the hydration shell of the lysozyme, and the fastest relaxing component T_{1f} belongs to strongly-bonded protons, e.g., those of internal (or crystallization) water [14, 29]. Note that the fastest contribution becomes progressively smaller at the highest temperatures and because the internal water exchanges with the hydration water when the protein unfolds, it has a weight of less than 10%. Note that although

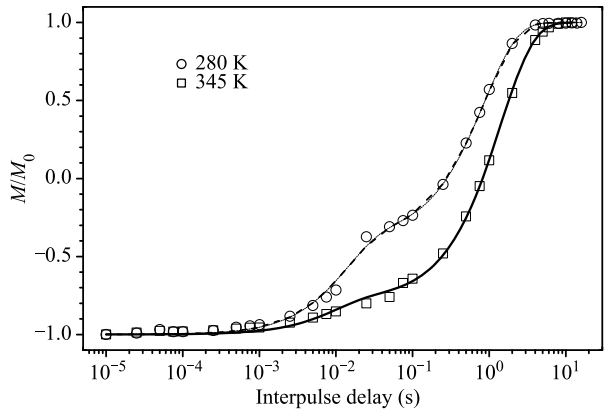

Fig. 1 Example of curve fitting after the execution of the inversion recovery pulse sequence for the determination of the spin-lattice relaxation time. The fit was performed with Eq. (1).

protein protons usually contribute to the NMR signal, under the experimental conditions in this study (and in particular low hydration), they are essentially immobile on the NMR observation time scale especially at low and ambient temperatures. Even with very high-resolution NMR techniques, at low hydration protein protons peaks are observable only for temperatures higher than the unfolding threshold and the magnitude of their intensity is about four orders smaller than that of the water peak [43]. In fact, in this case, in order to exclude the protein protons contribution, one can follow the relaxation corresponding just to the water peak.

3 Results and discussion

Although we know that the dynamics of water within the first hydration shell around the protein surface are slower than those of bulk water [45], the magnitude and molecular origin of this retardation are still topics for further study [46, 47, 52–55]. Fioretto *et al.* [52] used dielectric spectroscopy to measure the retardation coefficient, i.e., the ratio between the relaxation time of hydration water and the relaxation time of bulk water, and arrived at a value of 6–8 at ambient temperature [52].

Figure 2 shows in the bottom panel a log-linear plot of the ratio between the self-diffusion coefficient measured in bulk water [56, 57] and the self-diffusion coefficient measured in hydrated lysozyme with $h = 0.3$ versus the inverse of the temperature. The original data are reported in the top panel of Fig. 2; they were interpolated in order to calculate the ratio at the same temperature.

Although the value we find at ambient temperatures

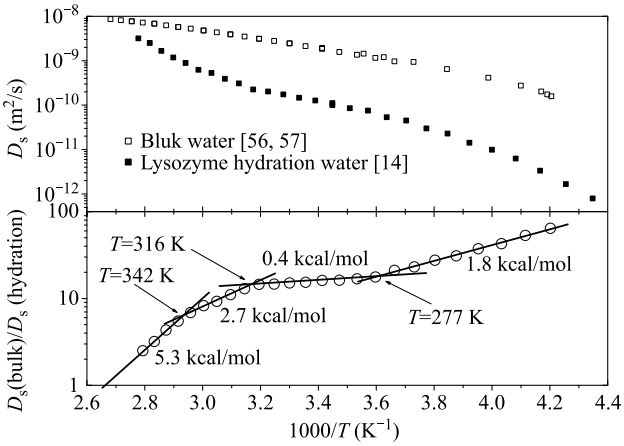

Fig. 2 The ratio between the self-diffusion coefficient measured in bulk water [56, 57] and that measured in hydrated lysozyme [43] as a function of the temperature (bottom panel), calculated from the interpolation of the original data reported in the top panel. Three variations are clearly visible in the obtained ratio.

is higher than that found by Fioretto et al., a more interesting observation is that the Arrhenius plot (see the bottom panel of Fig. 2) indicates that three changes occur at approximately 277 K, 316 K, and 342 K. By comparing the individual behavior, reported in the top panel of Fig. 2, one can note that the observed kink in the ratio originates from changes in the temperature evolution of the hydration water.

As we increase the temperature, the first change occurs at $T = 277$ K, which corresponds to the temperature at which bulk water has the maximum density. When $T < 277$ K, the activation energy is ≈ 1.8 kcal/mol, which is a typical hydrogen bond value. In this region the hydrogen bond network on the protein surface becomes progressively strong and stable. The region of optimal biological functioning between 277 K and 316 K (at which the second change occurs) shows a smaller activation energy (~ 0.4 kcal/mol). The ≈ 315 K temperature is the border temperature above which the hydrogen bond interaction weakens and water is no longer able to keep the protein in a folded state [39]. At temperature above 316 K, water is no longer a good solvent and the hydrogen bonded network of the first hydration shell begins to crumble and triggers the protein unfolding process. In the region between 316 K and 342 K, the activation energy is ≈ 2.7 kcal/mol and, depending on the duration, the unfolding process is reversible [43, 58]. At the highest activation energy (~ 5.3 kcal/mol), the unfolding process is irreversible. When $T > 342$ K, the hydrogen bond network is disrupted and the structure of the protein resembles that of a linear chain of amino acids. Note that the ratio between the self-diffusion coefficients of bulk and hydration water tends toward 1, which is the same as in bulk water for temperatures approaching the boiling point of bulk water. This implies that all water molecules are in a bulk condition and free to move.

Next, we examine the rotational dynamics by evaluating the reorientational correlation time using the measured self-diffusion and spin-lattice relaxation times. Assuming that the spin-rotation contribution to the proton relaxation rate is in the order of milliseconds at 360 K [59], and even shorter when below that temperature, only dipolar interactions can be taken into account for water relaxation and the observed proton relaxation can be written as the sum of inter- and intra-molecular contributions [60, 61],

$$\left(\frac{1}{T_1}\right)_{\text{obs}} = \left(\frac{1}{T_1}\right)_{\text{inter}} + \left(\frac{1}{T_1}\right)_{\text{intra}} . \tag{2}$$

The intermolecular term can be evaluated by using the self-diffusion coefficient D_s [60], as

$$\left(\frac{1}{T_1}\right)_{\text{inter}} = \frac{N\pi\gamma^4\hbar^2}{5aD_s}\left[1 + 0.233\left(\frac{b}{a}\right)^2 + 0.15\left(\frac{b}{a}\right)^4 + \cdots\right], \quad (3)$$

where N is the number density of the nuclei, γ the proton gyromagnetic ratio, $\hbar$ the Planck constant divided by 2π, a the molecular hydrodynamic radius, and b the distance of the proton from the center of the molecule.

At first approximation, for small time windows, the correlation function of protein hydration water can be considered exponential [62]. Therefore, in the extreme narrowing limit, i.e., when the product of the resonance frequency of the proton (ω_0) and the reorientational correlation time (τ_θ) is much less than one ($\omega_0\tau_\theta \ll 1$), it can be written as [60, 61],

$$\left(\frac{1}{T_1}\right)_{\text{intra}} = \frac{3}{2}\frac{\gamma^4\hbar^2}{r^6}\tau_\theta, \quad (4)$$

where r is the distance between the two protons in the water molecule. Thus, if we measure D_s and T_1, we can evaluate the intra-molecular term and the reorientational correlation time τ_θ. Because we are focusing on the hydration water, we evaluate τ_θ by considering the T_{1s} values and by assuming the constant values $a = 1.38$ Å, $b = 0.92$ Å, and $r = 1.52$ Å [60].

Figure 3 compares the thermal behaviors of τ_θ in lysozyme hydration water measured by NMR ($h = 0.3$), dielectric spectroscopy ($h = 0.37$) [24], and neutron scattering ($h = 0.4$) [25], with that of bulk water at ambient pressure [57] and emulsified water at 2 kbar [61]. Note that when the temperature drops below the temperature of irreversible denaturation, the reorientational correlation time in hydration water is slower than in bulk

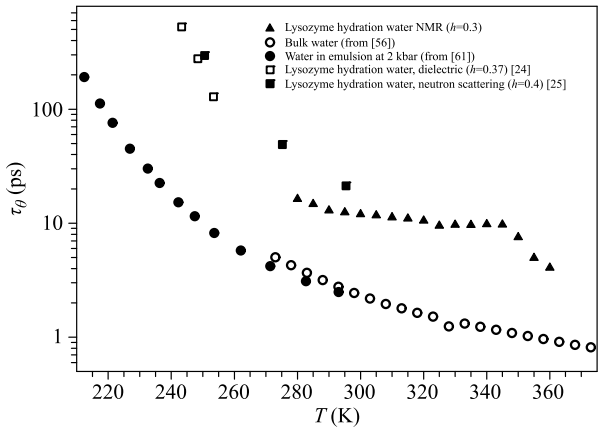

Fig. 3 The reorientational correlation time, τ_θ, of lysozyme hydration water measured by NMR ($h = 0.3$), dielectric spectroscopy ($h = 0.37$), and neutron scattering ($h = 0.4$), compared with that of bulk water at ambient pressure and to emulsified water at 2 kbar.

water, but when $T > 346$ K, the rotational dynamic values increase sharply and approach those of bulk water. This confirms the previous findings that when $T > 346$ K the hydrogen bonded network breaks down and water molecules move freely.

Our analysis can be used to differentiate between two different dynamical degrees of freedom of hydration water. The intra-molecular dynamics is linked with the single molecule rotation about its own axis (self rotation), whereas the inter-molecular dynamics depends on the water interaction with protein molecules. Because the orientational correlation time is in the order of picoseconds, water molecules on the protein surface are highly mobile; however, their diffusion is slowed down by their interaction with protein amino acids (resulting in an obstruction factor). By applying the Stokes–Einstein relation [63] we can estimate the hydrodynamic radius of the diffusing particles,

$$D_s = \frac{k_B T}{6\pi\eta\xi}, \quad (1)$$

where k_B is the Boltzmann constant, η the shear viscosity, and ξ the hydrodynamic radius (Fig. 4). It was shown that although the density of the first hydration shell of water around the protein surface is up to 20% higher than that of bulk water, the viscosity remains essentially the same [63]. Therefore, we evaluated the hydrodynamic radius by using the viscosity data of bulk water from the NIST database [64].

Note that when the temperature is low, the value of ξ (~ 1.8 nm) coincides with that of the hydrodynamic radius of lysozyme [54] confirming that the first hydration shell of water is hydrogen bonded with the hydrophilic groups of the protein surface. When $T > 320$ K, however, ξ rapidly decreases, the water molecules are no longer tightly bonded to the protein surface. They form local

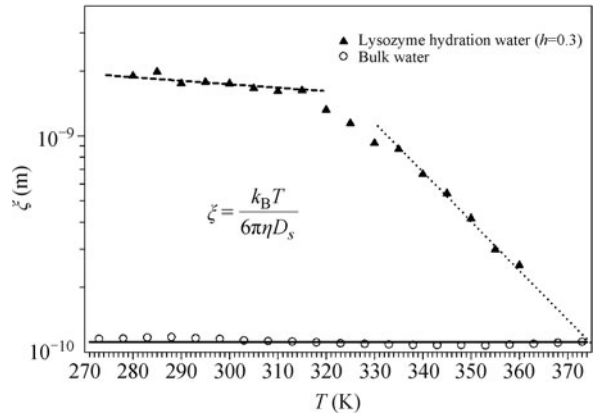

Fig. 4 The hydrodynamic radius obtained using the Stokes–Einstein relation for bulk water and lysozyme hydration water. Lines are guides for the eye.

and transient aggregates whose dimension progressively decreases as the temperature increases. Confirming this, when $T \gtrsim 345$ K and the protein unfolds completely, the ξ values of hydration water approach those of bulk water (dotted line in Fig. 4).

4 Conclusion

We studied the dynamics of water in the first hydration shell of lysozyme and linked the corresponding changes to different states of the folding/unfolding process. We used nuclear magnetic resonance spectroscopy to differentiate between the translational and rotational contributions, which are the proton self-diffusion coefficient and the reorientational correlation time, respectively.

The self-diffusion in the hydration water is slower than in the bulk solvent, and the ratio between these two values (a retardation coefficient) as a function of the inverse temperature, shows three changes at approximately 277 K, 316 K, and 342 K, with corresponding changes in the activation energy (see Fig. 2). When $T < 277$ K, the hydrogen bond network on the protein surface is strong and stable and the activation energy is ≈ 1.8 kcal/mol. The region between 277 K and 316 K is the thermal region of optimal biological functioning and exhibits a smaller activation energy (~ 0.4 kcal/mol). Above ~ 315 K, the hydrogen bonded network of the first hydration shell begins to crumble and the protein unfolding process begins. However, when the temperature is 316 K$< T < 342$ K and the activation energy is ≈ 2.7 kcal/mol, the unfolding process is still reversible [43, 58]. Finally, when $T \gtrsim 342$ K and the activation energy is ~ 5.3 kcal/mol, the unfolding process becomes irreversible, the hydrogen bond network breaks down completely, and the protein structure resembles that of a linear chain of amino acids.

In our rotational dynamics study, we found that for temperatures lower than the temperature of irreversible denaturation, the reorientational correlation time τ_θ in hydration water is slower than in bulk water (see Fig. 3). When $T \sim 346$ K, τ_θ sharply decreases and approaches the values for bulk water, and the water molecules can rotate freely.

Finally, we applied the Stokes–Einstein relation to the lysozyme hydration water and calculated the hydrodynamic radius of the particles (see Fig. 4). We found that at ambient temperatures the radius coincides with the lysozyme hydrodynamic radius (≈ 1.8 nm). When $T > 320$ K, ξ decreases sharply and when $T > 345$ K its value approaches that of bulk water. This result confirms that when lysozyme unfolds completely, the water molecules are essentially free and no longer bridge the protein residues.

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