IL NUOVO CIMENTO **39 C** (2016) 308
DOI 10.1393/ncc/i2016-16308-8

COLLOQUIA: SWGM 2015

The time dependence dynamics of hydration water changes upon crossing T^*

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received 19 July 2016

Summary. — We carry out a Nuclear Magnetic Resonance (NMR) spectroscopy study on the dynamics of lysozyme hydration water. We consider a hydration level corresponding to a single water monolayer. We investigate the thermodynamical region from 295 K to 355 K, at temperatures below and above the "magic" temperature $T* \approx 320$ K. In particular, we focus our attention on hydration water mean-square displacement (MSD) as a function of the diffusion time at different temperatures. Our results suggest the occurrence of a smooth anomalous diffusion from a sub-diffusive state ($T < T^*$) to a super-diffusive one ($T > T^*$). These conclusions confirm the importance of the temperature T^* as the border for water behavior.

1. – Introduction

Liquid water is essential for life processes in all biological systems [1-3]. Probably the most important ability of water is to form the so-called hydrogen bonds (HBs). In fact, HBs are responsible for water well-known thermodynamic anomalies that determine its properties in both the stable and the supercooled phases [4, 5]. Note that the HB lifetime strongly depends on the thermodynamical conditions, such as temperature, pressure, etc. [6]. In particular, several studies have shown that there is a "magic temperature", identified as $T^* \approx 320$ K that signs a crossover from a simple to a complex liquid behavior, characterized by the water HB clustering [4]. In the frame of the dynamics of water-protein systems, HB interactions drive the structure, dynamics, and functioning of biological macromolecules [7]. Indeed, the study of hydration water is

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key to understanding the essential processes that occur in proteins activity. In fact, the physical and chemical properties of proteins depend on the attribute of HBs formed between the protein residuals and with its hydration water [8]. Several studies using various techniques have been carried out on hydrated biological macromolecules in different thermodynamical conditions [8-20] confirming the importance of HBs and the role of the temperature in these kinds of systems. Therefore, T^* represents an important temperature also for hydrated proteins [8,20,21]. In fact, proteins are in their native and stable state for temperatures below T^*. Above this temperature, there is an enhanced decreasing of HB lifetime and this does not allow to keep together the protein residuals giving rise to the unfolding process [21]. Hence, above T^*, a protein passes through an intermediate state and the folding-unfolding process is somehow reversible [8]. Above the threshold of irreversible denaturation, when the protein unfolds completely, it loses its characteristic three-dimensional folded state that corresponds to its peculiar enzymatic activity. Note that the alteration of the structure of proteins due to incorrect folding (or misfolding) process is the source of neuro-degenerative illnesses such as Alzheimer's and Parkinson's diseases [22]. In this paper, we study hydrated lysozyme by focusing our attention just on the high-T region, in which the folding-unfolding process occurs. For lysozyme, the reversible folding-unfolding process starts just at $T^* \approx 320\,\mathrm{K}$ and the irreversible denaturation takes place at $T_D \cong 345\,\mathrm{K}$ [8, 15, 16, 23, 24].

It is clear how important can be the study of the properties of these kind of systems. In particular, the HB dynamics can be investigated and studied in details by measuring the water self-diffusion coefficient. Indeed, translational diffusion is one of the most fundamental transport processes in chemical and biochemical systems [25, 26]. Self-diffusion D_S is the random translational motion of molecules (or ions) driven by internal kinetic energy. It is a spontaneous mixing of molecules taking place in the absence of concentration (or chemical potential) gradient. Diffusion is closely related to molecular size, by the Stokes-Einstein equation $D = \frac{kT}{f}$, where k is the Boltzmann constant, T is temperature, and f is the friction coefficient.

Because of its non-invasive nature, nuclear magnetic resonance (NMR) spectroscopy is a unique tool for studying molecular dynamics in chemical and biological systems [25,26]. In this work, we use the Pulsed-Field gradient stimulated echo (PFG-STE) Nuclear Magnetic Resonance (NMR) technique [27,28] to measure the mean-squared displacement (MSD) of lysozyme hydration water. In this frame, the study of translational diffusion measurements can be associated with a characteristic timescale Δ (an "observation" or "diffusion" time). In the case of homogeneous solution and without chemical exchange, the duration of the diffusion time (Δ) is not relevant and we get the same result of the Brownian condition (*i.e.*, the MSD scales linearly with time) [25, 27]. Instead, when a species diffuses within a confined space, the displacement along the z-axis of the diffusing particle will be a function of Δ, of the diffusion coefficient, and of the size and of the shape of the restricting geometry [29]. In this case, we are in the presence of the so-called "anomalous diffusion" [27,30,31]. In a water-protein system, the scenario is rather more complex because the probe molecule (in this case, water) interacts with the larger macromolecules (*e.g.*, proteins) [32]. These interactions act on a time scale much smaller than the smallest experimentally available Δ and, thus, they can be correctly averaged on the time scale of Δ [27].

The aim of this paper is to study the dependence of MSD for lysozyme hydration water on the diffusion time Δ at temperatures below and above the "magic" temperature T^* in order to check if we are in the presence of anomalous diffusion.

2. – Materials and methods

Lysozyme is a small globular protein enzyme composed of 129 amino acid residues. Lysozymes act as part of the body's defense system against bacteria and they can be found in tears, human milk, saliva, and mucus. The ability of lysozyme to break down bacterial cell walls in order to improve protein and nucleic extraction efficiency make this protein a very important macromolecules in living organisms. Generally, when a protein interacts with water, its biochemical activity is strongly influenced by the hydration level h, *i.e.*, the grams of H_2O per grams of dry protein [18, 33]. For lysozyme, the enzymatic activity is very low up to $h \cong 0.2$, 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 (the first hydration shell) [34]: in this situation, the protein functionality is activated. Furthermore, it has been proved that this shell remains liquid at all hydrations, so it plays a bioprotective role at low temperature since it protects the functioning of the protein and disallows irreversible changes in the molecular structure [18].

In this work, we study the dynamic properties of lysozyme hydration water at different temperatures (295 K, 315 K, 335 K, 355 K) and at ambient pressure using a Bruker AVANCE NMR spectrometer operating at 700 MHz ^{1}H resonance. We measure the MSD of the molecules for several values of diffusion time Δ. This approach takes into account the fact that complex materials can exhibit generalized Brownian motion (*i.e.*, Lévy flight or fractal diffusion) rather than simple Brownian motion [35, 36]. In our experiment, we use the Pulsed-Field gradient stimulated echo technique (^{1}H PFG-STE) and a powder of the globular protein lysozyme hydrated with $h = 0.3$ according to a precise procedure [11].

In the PFG method, the attenuation of a spin-echo signal results from the dephasing of the nuclear spins due to the combination of the translational motion of the spins and the imposition of spatially well-defined magnetic field gradient pulses. This is used to measure the displacement of the observed spins allowing to distinguish chemically and physically completely identical species (*e.g.*, in the liquid phase, as for example water molecules within liquid water) [25, 27].

In the PFG-STE technique, a first 90° pulse excites the magnetization, that is then phase dispersed by a magnetic field gradient pulse. As shown in fig. 1, a second 90° radiofrequency pulse is applied that moves half of the dispersed magnetization onto the z-axis. After a delay that allows diffusion to occur, a third 90° radiofrequency pulse returns the dispersed magnetization to the x, y-plane where a final magnetic field

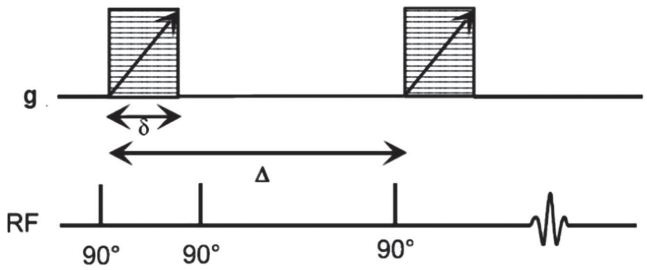

Fig. 1. – Diagram of the PFG-STE pulse sequence. RF indicates the radio frequency, Δ is the diffusion time, δ and g are the duration and the magnitude of the gradient pulse, respectively.

gradient pulse refocuses it to yield a signal whose intensity is dependent on the diffusion rate [27, 28, 37, 38].

To relate diffusion to the attenuation of the echo signal and to the experimental parameters in the PFG NMR experiment, we can use the Gaussian phase distribution (GPD) approximation and the short gradient pulse (SGP) approximation (described in details in [25]). In fact, in a homogeneous system, the diffusion propagator [27, 28, 39] can be assumed Gaussian and the echo attenuation due to relaxation, obtained from the PFG-STE technique within the Short Gradient Pulse (SGP) limit [25, 27, 40], can be described using the following equation:

$$\Psi(\Delta) = \Psi_0 \exp[-(2\pi q)^2 \langle z^2(\Delta)\rangle/2] = \Psi_0 \exp[-(\gamma\delta g)^2 D\Delta)], \tag{1}$$

in which Δ is the diffusion time, γ is the nuclear gyromagnetic ratio, a constant for each type of nucleus, δ and g are the duration and the magnitude of the gradient pulse, respectively. In our experiment, we use $3 < \Delta < 40\,\text{ms}$, a fixed $\delta = 0.2\,\text{ms}$, and $0 < g < 1200\,\text{Gauss/cm}$. The duration of the 90° pulse is $22\,\mu\text{s}$ and the repetition time is 10 s. Furthermore, the quantity $\gamma\delta$g in eq. (1) corresponds to the "NMR generalized" scattering vector q and that the mean-squared displacement along the z-axis, $\langle z^2(\Delta)\rangle$, is equal to $2D\Delta$.

In heterogeneous media, the diffusion propagator can no longer be considered Gaussian and the echo can be described, for example, by a stretched exponential [40, 41]. In general, higher-order moments will then have to be included in eq. (1), and this will make it complicate to obtain important informations about the investigated media. However, to be able to analyze the PFG NMR data from heterogeneous systems, it is possible to consider the second cumulant approximation [28]. Using this approach, the initial decay of eq. (1) is mostly dependent on the lowest order of g (g^2 for a Gaussian propagator). As the contribution due to higher orders of g is much smaller than the contribution obtained from the second order, the initial decay should reflect a Gaussian distribution [39]. In this situation, as reported in ref. [42], the spin echo attenuation is a polynomial of even powers in q multiplied by even cumulants and, precisely, Ψ is the second power in q multiplied by the mean-squared deviations from the mean displacement. With this in mind and starting from eq. (1), we have performed a linear fit in the initial part of the log-log graph of the spin echo attenuation of the NMR signal for each temperature measured to get the MSD along the z-axis [36].

Note that, in the case of inhomogeneous systems and, hence, of "anomalous diffusion", the MSD can be described by a power law [26, 40, 43, 44]

$$\langle z^2(\Delta)\rangle = 2D\Delta^{\alpha}, \tag{2}$$

in which α is the so-called fractal diffusion exponent [36]. In a typical Brownian diffusion process, $\alpha = 1$. If $\alpha > 1$, the phenomenon is called super-diffusion and if $\alpha < 1$, the particle undergoes sub-diffusion [26, 44-48]. In this frame, we have executed a linear fit using the equation $\ln\langle z^2(\Delta)\rangle = \ln(2D) + \alpha \ln\Delta$ to calculate the alpha coefficient for all the studied temperatures.

3. – Results and discussions

Figure 2 shows the normalized measured spin-echo attenuation in a log-log plot (*i.e.*, Ψ/Ψ_0 *vs.* $q^2\Delta$, at different observation times) for hydrated lysozyme ($h = 0.3$) at

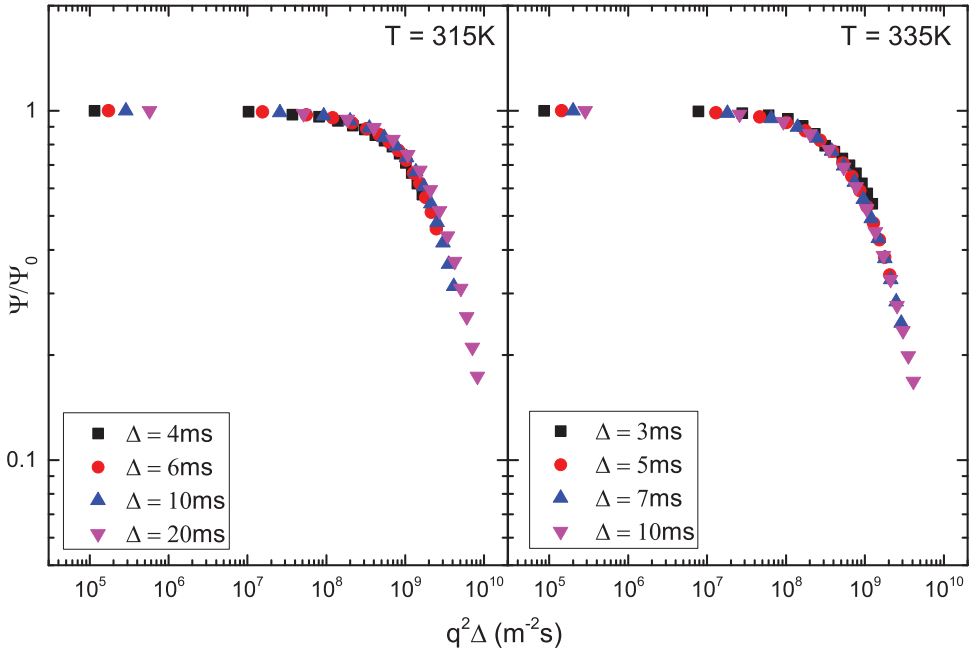

Fig. 2. – The normalized pulsed gradient spin-echo NMR attenuation Ψ/Ψ_0 *vs.* the reduced variable $q^2\Delta$ (at different Δ values and same gradient pulse width δ), for hydrated lysozyme at the temperatures 315 K and 335 K, *i.e.*, below and above T^*, respectively.

temperatures 315 K and 335 K, below and above the magic temperature T^*, respectively. Note that each spin-echo attenuation data are obtained for different Δ's at a fixed $\delta = 0.2$ ms.

Note that all the data overlap to each other indicating a not well evident dependence on diffusion time. As mentioned before in "Material and methods" section, for the second cumulant approximation [28, 42], a spin-echo attenuation signal can be well described at low q by an exponential with the form of eq. (1).

In fig. 3, we show the comparison between the spin-echo attenuations for all the investigated temperatures. In particular, in the figure, we report data with a diffusion time Δ of 4 ms for $T < T^*$, instead, for T above the magic temperature, $\Delta = 3$ ms. The solid curves represent fits of the data with eq. (1). We performed the best-fit procedure in a constant data range of $1 > \Psi/\Psi_0 > 0.8$. Note that the linear trend holds for all the q^2 range only for the lowest temperature. The deviation from linearity is strongly enhanced for the temperature above the denaturation threshold.

We identify this behavior as due to the progressive unfolding of the protein with the subsequent "release" of the "internal water" that mixes up with hydration water [16] and now contributes to the dynamics. In fact, in hydrated proteins, two types of water can be identified: bound internal water and hydration water. The first one has molecules located in internal cavities and fractures playing a structural role in the folded protein itself. Instead, hydration water is the first water layer and it strongly interacts with the protein surface, as if water is confined, controlling the biofunctionality of the protein [18]. Furthermore, water can influence both the hydrophilic and hydrophobic

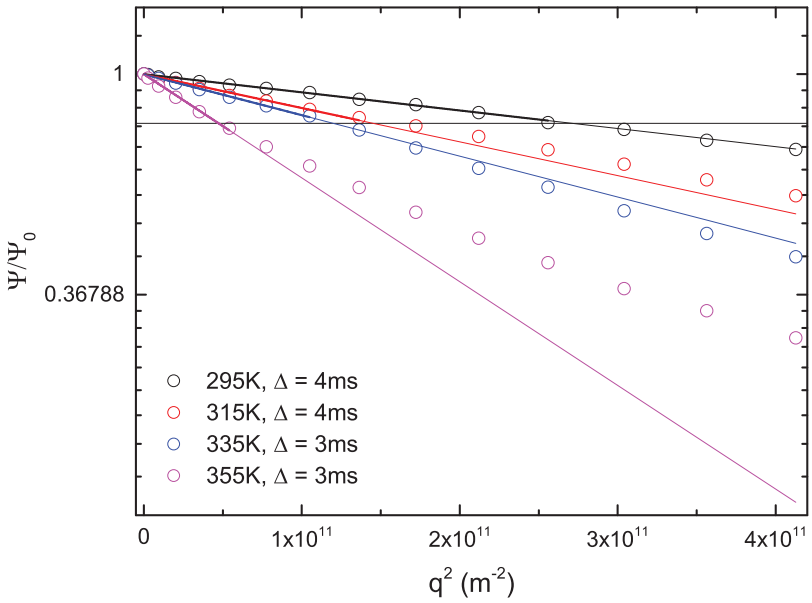

Fig. 3. – The normalized spin-echo amplitude Ψ/Ψ_0 *vs.* q^2 for the investigated system at the indicated diffusion times and temperatures. The solid curves represent fits of the experimental data with eq. (1).

groups of a biomolecule. Because the properties of the surface water strongly influence protein stability and function, studying how the hydrophilicity (the HB strength) governs the secondary structure and folding specificity of the biomolecule is a very important topic [20].

From the fits of fig. 3, we get the MSD, and we report the obtained $\langle z^2(\Delta)\rangle$ values in fig. 4, as a log-log plot at the temperatures 295 K, 315 K, 335 K and 355 K. From fig. 4, we notice that the slope (α) in eq. (2) increases with temperature, indicating the presence of anomalous diffusion. This is due to the fact that, on increasing the temperature, the HBs can be easily broken causing a greater mobility to the protein side-chains. Therefore at the highest temperatures internal water becomes essentially free, can diffuse and interact with hydration water [16]. Indeed, considering eq. (2), in fig. 5 we report the α values for all the temperatures and the Δ's as fit results of $\langle z^2(\Delta)\rangle$. Here, we show the dependence of the exponent α of anomalous diffusion on temperature: results demonstrate that α increases as the temperature increases, indicating, as mentioned before, an enhancement of protein chain mobility as the system performs a transition from the folded to the unfolded state. In particular, the system passes from a condition in which $\alpha < 1$ (the particle undergoes sub-diffusion) to $\alpha > 1$ (super-diffusion). The onset of this change in the diffusivity of the studied system can be recognized in the magic temperature $T^* \approx 320\,\mathrm{K}$. In fact, for $T \approx T^*$, α is around 1 and the motion is Brownian.

4. – Conclusions

In this work we have studied the changes in the diffusivity of protein hydration water with the temperature and diffusion time Δ. To this aim, we perform several

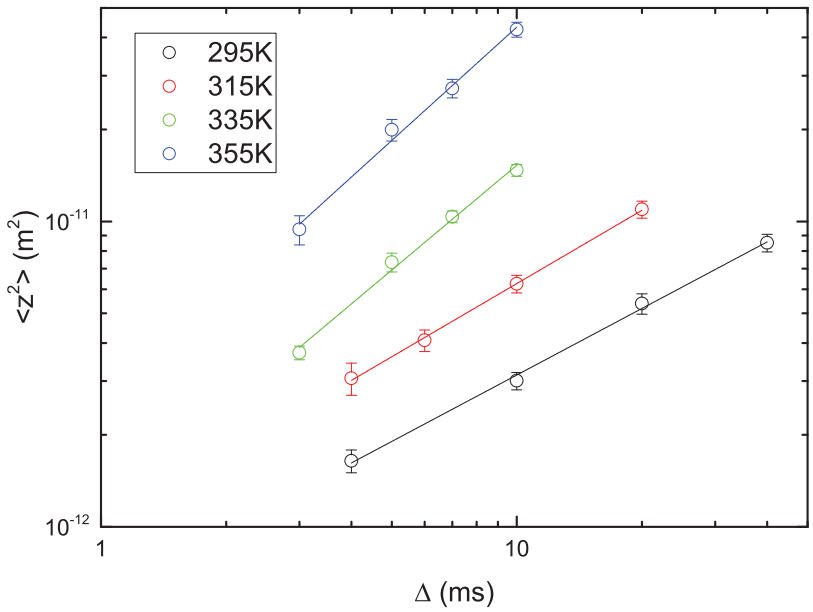

Fig. 4. – Mean-square displacements as a function of observation time Δ for hydrated lysozyme at the temperatures indicated.

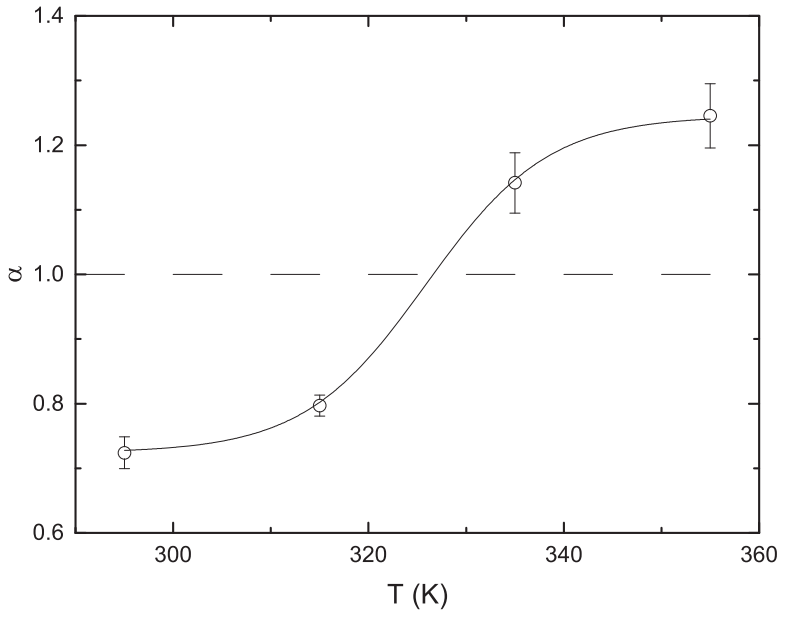

Fig. 5. – T dependence of the power law exponent α for hydrated lysozyme. The system goes from a sub-diffusive state ($\alpha < 1$) to a super-diffusive one ($\alpha > 1$) upon crossing the magic temperature T^*.

measurements by means of PFG-STE NMR spectroscopy at different temperatures (295 K, 315 K, 335 K and 355 K) to investigate the region of folding-unfolding transition. Figures 2 and 3 show the spin echo attenuation data. We notice that the curves follow a precise trend indicating a non-clear dependence on diffusion time. The decay of the spin echo attenuation exhibits an increasing feature of nonlinearity as the denaturation region is approached ($T > 345$ K). The profile of the decay can be described by an exponential with the form of eq. (1) and we notice the presence of moderate anomalous diffusion: in fact, as shown in fig. 4, the mean-square displacement $\langle z^2 \rangle$ shows a power law dependence on observation time Δ (see eq. (2)). We point out that all these considerations lead us to the following statement: by increasing the temperature and when protein reaches the unfold condition, the protein side-chain obtains a greater mobility due to the higher probability of HB breaking. The value of the anomalous diffusion exponent α, reported in fig. 5, increases with temperature and there is a crossover at T^* in which $\alpha \approx 1$. This temperature represents the "magic point" at which the system goes from a condition in which $\alpha < 1$ (sub-diffusion) to $\alpha > 1$ (super-diffusion). Such a picture indicates the importance of T^*, not only in water, but generally in water systems. In particular, our data confirm that, for $T < T^*$, water is able to form stable HBs, whereas for $T > T^*$, HBs lifetime and strength are much smaller and water behaves as a simple liquid.

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The authors acknowledge the Consiglio Nazionale delle Ricerche for the support. DM's activity is carried out within the framework of the NANORESTART project which has received funding from the European Unions Horizon 2020 research and innovation programme under grant agreement No. 646063.

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