Role of the solvent in the dynamical transitions of proteins: The case of the lysozyme-water system

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We study the dynamics of hydration water in the protein lysozyme in the temperature range $180\ \mathrm{K} < T < 360\ \mathrm{K}$ using Fourier-transform-infrared and nuclear magnetic resonance (NMR) spectroscopies. By analyzing the thermal evolution of spectra of the OH-stretching vibration modes and the NMR self-diffusion (D_S) and spin-lattice relaxation time (T_1), we demonstrate the existence of two dynamical transitions in the protein hydration water. Below the first transition, at about 220 K, the hydration water displays an unambiguous fragile-to-strong dynamic crossover, resulting in the loss of the protein conformational flexibility. Above the second transition, at about 346 K, where the protein unfolds, the dynamics of the hydration water appears to be dominated by the non-hydrogen-bonded fraction of water molecules. © *2007 American Institute of Physics*. [DOI: 10.1063/1.2757171]

I. INTRODUCTION

Understanding the relationship between the structure and dynamics of proteins[1] and the water associated with them is an ongoing challenge.[2–4] Without water a protein cannot function but a single layer of water surrounding it (called the first hydration layer) restores activity,[1,5] so biological functions[6] such as enzyme catalysis can only be understood with a precise knowledge of the structure and function of the first hydration layer. When a protein is in solution, there are two categories of water molecules identifiable in close proximity to the protein: (i) the bound internal water, and (ii) surface water usually called hydration water. The bound internal water molecules, located in the internal cavities of the protein, play a structural role in the folded protein itself. At low temperatures, a protein exists in a state[7,8] without conformational flexibility. As T increases, the atomic motional amplitude initially increases linearly, as in a harmonic solid. In hydrated proteins, at about 220 K, the rate of amplitude increase with temperature suddenly becomes enhanced, signaling the onset of more liquidlike motion.[9–11] This "dynamical transition" of proteins may be triggered by coupling of the protein with the hydration water through hydrogen bonding, since protein hydration water shows a dynamic transition at a similar temperature.[12]

Another phenomenon governing biological properties of proteins occurs at high temperatures, just below the onset of protein denaturation. A protein is in the native state up to a given temperature and evolves, on increasing T, into a region characterized by a reversible unfolding-folding process. The phenomenon depends on the chemical nature of the protein and the solvent. In the case of the system water lysozyme such a phenomenon occurs in the temperature range of 310–360 K. Above 355 K, lysozyme denatures irreversibly. For such a system, calorimetric measurements[13] show a broad peak in the specific heat around that temperature. The rate constant varies with T according to an Arrhenius law,

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with an activation energy typical of the strength of the hydrogen bond (HB),[13] so hydration water appears to play a determinant role also for this transition.

Although the two transitions are known, in order to have a more complete understanding, an increasing attention is being devoted to the properties of hydration water. Instead of behaving like other simple molecular liquids, water displays counterintuitive trends in many of its thermodynamic response functions and transport coefficients. There are two thermodynamically consistent hypotheses that try to account for the water's anomalies.[3] One hypothesis under investigation, favored by preponderance of evidences, is that water has, in addition to the vapor-liquid critical point a second critical point below which two distinct liquid phases coexist: the liquid-liquid (LL) critical point hypothesis.[14–17] According to this hypothesis, below the second critical point, two distinct phases appear, the low-density liquid (LDL) and the high-density liquid (HDL). In the LDL, an open, locally ice-like, HB network appears[1–3] whereas in the HDL, the local tetrahedrally coordinated HB network is not fully developed. The structure of the LDL phase is not unlike that of the well-studied low-density amorphous (LDA), while the structure of the HDL phase is not unlike that of the high-density amorphous (HDA) solid. The phenomenon of a phase transition between two amorphous phases differing in their respective densities and entropies is known as polyamorphism.

Above the LL critical point, water is in one phase and, like all critical points, there is a Widom line (locus of maximum correlation length) extending into the one-phase region. On the high-temperature side of the Widom line, the local structure resembles that of the HDL liquid phase, while on the low-temperature side it resembles the LDL liquid phase.

The LL critical point hypothesis has received strong support from recent studies[18–20] on water confined in nanopores. In a series of neutron scattering and NMR spectroscopy experiments, confirmed by molecular dynamics simulations, a fragile-to-strong dynamic crossover[21] (FSC) and a violation of the Stokes-Einstein relation[22] occur at about the same temperature $T_L \sim 225$ K.[23] Furthermore, simulations demonstrate that the locus of FSC in the P-T phase diagram coincides with the Widom line.[18,20] The neutron scattering experiments also locate the end point of the Widom line, which is the LL critical point.[18] Additionally, Fourier-transform-infrared (FTIR) spectroscopy in the temperature range of 273–183 K demonstrates that LDL is continuous in structure with LDA (Ref. 24) as predicted by the LL critical point hypothesis.

As a further test of this hypothesis, high-resolution quasi-elastic-neutron scattering experiments on protein hydration water demonstrate that the low temperature dynamic transition of lysozyme protein coincides with the FSC (at about 220 K), similar to that recently observed in water confined in nanopores.[25] Computer simulations of protein hydration water[26] locate the Widom line and demonstrate that the same anomalies found in experiments occur when the system crosses the Widom line.

Here we deal with the dynamics of the hydration water in a powder of the globular protein lysozyme. The hydration has a strong influence on protein dynamics, as demonstrated by experiments and simulations,[27] and biochemical activity. In lysozyme, enzymatic activity remains very low up to a hydration level h of about 0.2 (h is measured in grams of water per grams of dry protein) then increases sharply when h increases from 0.2 to 0.5.[28] Proteins also show a sharp slowing down in their functionalities as a transition temperature, generally within about 10% of 225 K, is approached. A quantity characterizing this phenomenon in proteins is the mean-squared atomic displacement $\langle x^2 \rangle$; at low T, $\langle x^2 \rangle$ varies linearly with T, whereas above the transition temperature it increases sharply. Neutron scattering experiments 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.[25]

Motivated by these findings, we report here results of FTIR and NMR experiments on hydrated lysozyme, which demonstrate that hydration water plays a role in the protein dynamical transition. In particular, our results are consistent with the picture that the dynamic behavior associated with the protein glass transition can be ascribed to fully hydrogen bonded molecules while the dynamics of water at the reversible folding-unfolding process is dominated by nonhydrogen bonded water molecules.

II. RESULTS AND DISCUSSION

Figure 1(a) shows the experimental FTIR OH stretch (OHS) spectra of hydration water for lysozyme with hydration level $h=0.3$. We also measured the same vibrational spectra for $h=0.37$ and 0.48. From spectroscopic methods[29–31] we have clear evidence that the physics of water is characterized by the existence of two coexisting main hydrogen bonded structural phases. In particular, the observed presence in both Raman and FTIR OHS spectra of a precise isosbestic point constitutes a strong evidence of a "mixture model" of water involving HB and non-hydrogen-bonded (NHB) molecules.[32] The same picture emerges from the Errington-Debenedetti analysis,[33] where the distribution of tetrahedralities is likewise bimodal and has isosbesticlike behavior. On these bases it became customary to analyze OHS spectra by considering two general classes of OH oscillators. These classes, however, encompass broad Gaussian components, each one referring to structures which involve a range of bond angles and distances distributed around the component peak position. Our data analysis was done considering such a situation[29,32] and the result coming out from recent FTIR experiments, in which we have discovered a novel Gaussian contribution related to the LDL phase.[24] We analyzed the measured OHS spectra [Fig. 1(b)] in terms of three main contributions. One arises from LDL, centered at about [(III) 3100 cm^{-1}], and the other two from HB [(I) $\sim$3220 cm^{-1}] and NHB (II) water molecules. As can be observed in Fig. 1(b), the LDL contribution (III) seems to play a determinant role below T_L whereas at the highest T the NHB component (II) is dominant. Such a situation is well represented in Fig. 2(a) which reports the relative weights (integrated area) of these three OHS components, for the

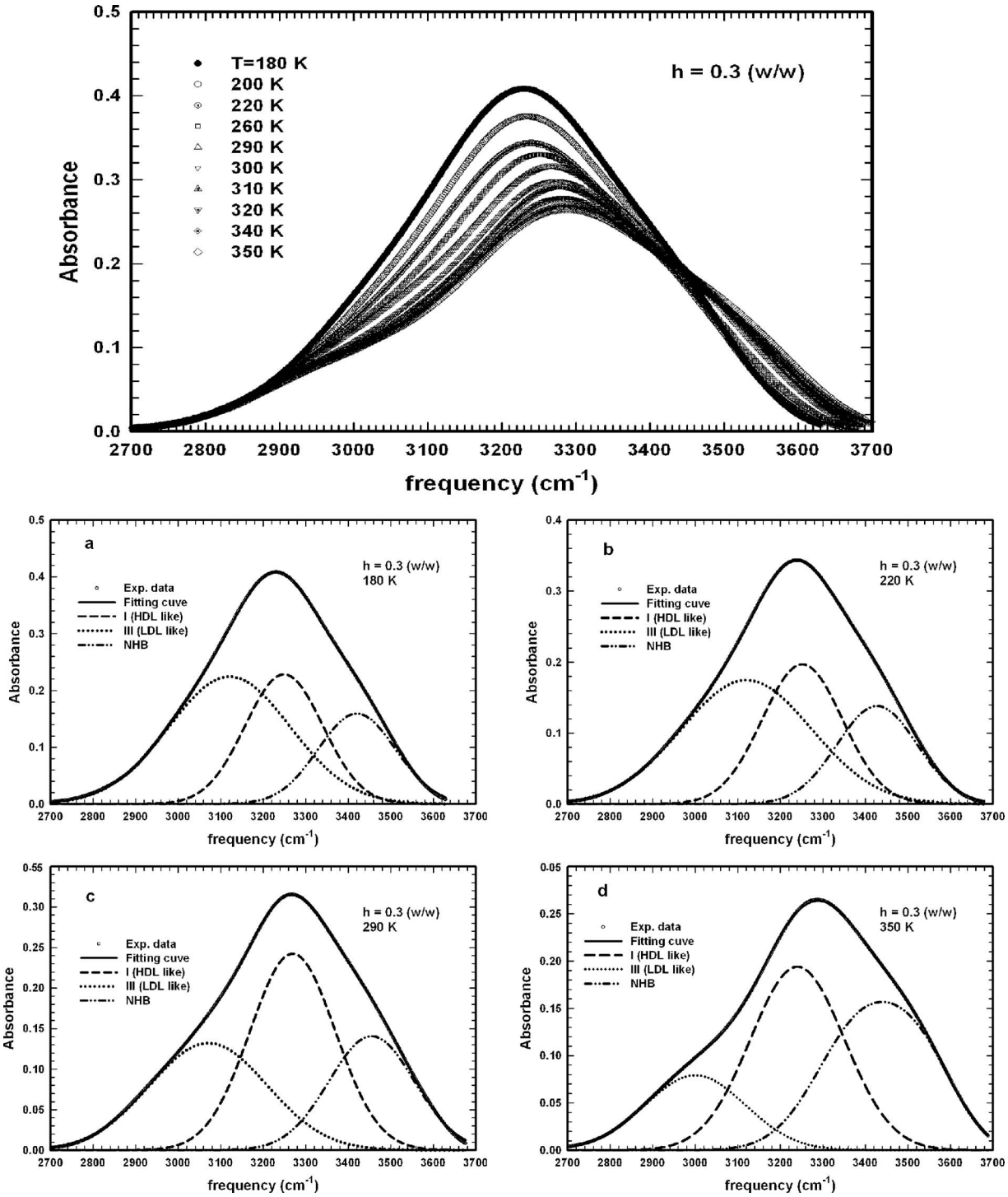

FIG. 1. (a) OH stretch (OHS) bands, in the 2700–3800 cm^{-1} range, of lysozyme hydration water at hydration level $h=0.3$, measured at different temperatures. As it can be observed their intensity increases by decreasing temperature; the three most intense ones correspond to a temperature below the FSC, whereas the three of lower intensity belong to the region in which protein denaturation takes place. (b) The deconvolution of the OHS spectra at $T=180$, 220, 290, and 350 K. In all the figures, dots represent the experimental data, and the solid lines are the best fits. The dotted and dashed lines are the contributions to OHS oscillators from the low-density water (LDL) and the hydrogen-bonded molecules (HB), respectively. The dash-dot-dot lines indicate the spectral contributions of the non-hydrogen-bonded molecules (NHB). The dotted line refers to component III centered at 3100 cm^{-1}, and the dashed line to component I centered at 3220 cm^{-1}.

three different measured hydration levels ($h=0.3$, 0.37, and 0.48). However, the remarkable result shown in Fig. 2(a) is that there are two main crossovers in the population of the three species of oscillators (areas): a low-temperature transition at about T_L (the protein dynamical transition and the FSC) and a high-temperature transition at T_D (temperature of the maximum C_P, which is inside the folding-unfolding reversible region and below the temperature of irreversible denaturation). The low temperature transition is due to the crossing between fraction of LDL phase (which increases on lowering T) and that of the HB phase, which decreases. The high-temperature transition appears on increasing T, from the

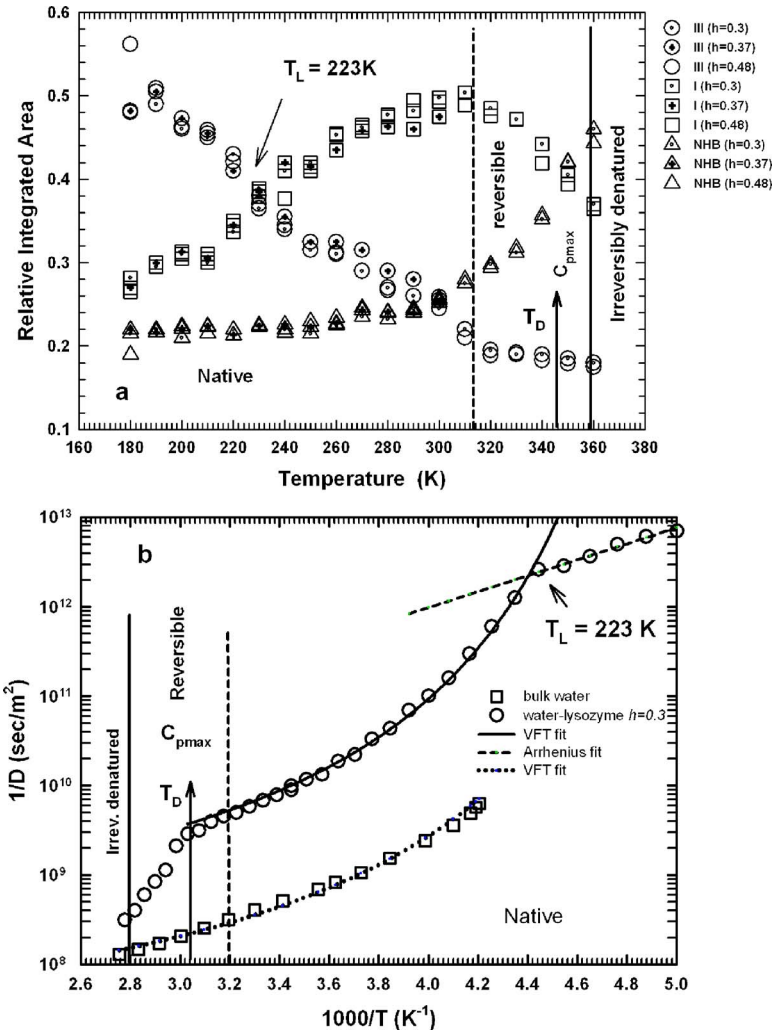

FIG. 2. (a) Relative integrated areas of the three FTIR components at three different lysozyme hydration levels, $h=0.3$, 0.37, and 0.48. Squares indicate the fractional contribution of component I at 3220 cm^{-1}, circles the fractional contribution of component III ("LDL") at 3100 cm^{-1}, and triangles the non-hydrogen-bonded (NHB) water. The three different regions of lysozyme behavior are also indicated, namely, native, reversible unfolding, and irreversible denaturation. Arrows show the temperature of maximum specific heat T_D (Ref. 13) and the FSC crossover temperature T_L. As one can see, the NHB and HB relative areas cross just near T_D. (b) The inverse of the NMR self-diffusion coefficient D of lysozyme hydration water as a function of 1/T (circles). Squares represent the values measured in bulk water. In the native region, $1/D$ of both bulk water and lysozyme hydration water obeys to a VFT law, but the protein hydration water displays a transition to an Arrhenius behavior at the FSC temperature $T_L \sim 223$ K. The location of this crossover temperature agrees with earlier neutron scattering experiments (Ref. 25) giving the temperature at which the protein looses its function. At a higher temperature (T_D), where the denaturation process of the protein takes place, the NMR self-diffusion data also show a second dynamical transition of hydration water.

crossing between the increasing population of the NHB phase with the one of the HB. Both these results demonstrate the role of water in determining the protein dynamics.

Figure 2(b) shows the inverse of the NMR measured self-diffusion constant D of hydration water molecules as function of $1/T$, for $h=0.3$, compared with that of bulk water. The thermal behavior is the same, and both the water species follow a Vogel-Fulcher-Tamman (VFT) law, $1/D = 1/D_0 \exp(BT_0/(T-T_0))$. For bulk water, the ideal glass transition temperature is $T_0=175$ K, whereas for the protein hydration water $T_0=182$ K. However, there is a factor of 10 between the $1/D$ of bulk water and that of protein hydration water.

Also in the behavior of $1/D$, two main crossovers can be observed. One crossover is at high T, where the protein changes from its native state to its unfolded state. On increasing T, $1/D$ decreases toward the value of pure bulk water. The second crossover takes place at $T_L=223$ K, location of the FSC, thus fully confirming the neutron scattering results on the same system.[25] The activation energy of the Arrhenius process in the strong region is $E_A = 3.48$ kcal/mol, whereas the neutron experiment gives 3.13 kcal/mol. Thus also these D data show unambiguously that T_L is the temperature characterizing the protein dynamical transition.

To get further information on the dynamics of hydration water in the high-temperature crossover (characterizing the onset of reversible folding-unfolding as shown by calorimetry[13]), we also measured the NMR proton spin-lattice relaxation time constant T_1 of the lysozyme hydration water with $h=0.3$ in the temperature interval $275 < T < 355$ K (Fig. 3). T_1 represents the longitudinal relaxation time of protons, and is connected, together with the spin-spin proton relaxation time T_2 (transverse relaxation), to the transport properties of the system.[34] Figure 3 also shows T_1 for pure bulk water. We see that the hydration water spin lattice relaxation time is characterized by two contributions, one coming from the hydration water protons and the other one from the protein protons. The first T_1 is of the order of seconds (as in bulk water), whereas the second one is of the order of 10 ms. Moreover, the figure shows that on increasing T, the bulk water T_1 follows the VFT law in the whole studied temperature interval. Instead, the T_1 of hydration water is characterized by two different behaviors above and below the onset of the reversible unfolding regime. In the protein native state, the T_1 of hydration water increases with T, following a behavior that is similar to that of bulk water, whereas the T_1 of the protein protons remains nearly constant. The situation changes dramatically when T approaches the region of the high-temperature protein dynamical transi-

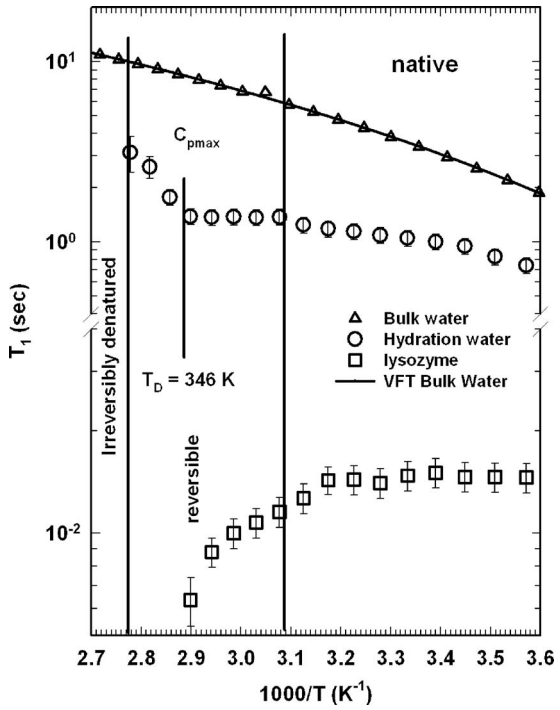

FIG. 3. NMR longitudinal (spin-lattice) relaxation time T_1, as a function of $1/T$, for temperatures above and below the region of protein denaturation. Triangles correspond to bulk water, squares to protein hydration water, and circles to protons in the protein. Also for such a dynamical parameter, dramatic changes in the case of hydration water and protein protons may be observed within the denaturation temperature region.

tion: the proton spin-lattice relaxation time of the protein protons drops abruptly and disappears just at T_D. Conversely, the T_1 of hydration water remains nearly constant, and afterwards it shows a sudden increase toward the values of bulk water, before irreversible denaturation intervenes. This behavior is analogous to that of the self-diffusion coefficient. In summary, also in the high temperature region, there is an overall agreement between the NMR data ($1/D$ and T_1) and the FTIR OHS vibrational spectra results [Fig. 2(a)]. The protein denaturation process, accompanied by an early stage of reversibility starts just when the population of NHB molecules approaches that of the HB ones, i.e., just when the probability for water molecules to form a HB is about the same of that to be nonbonded.

III. CONCLUSIONS

In conclusion, the investigation of the population of the differently HB and NHB water molecules by means of FTIR spectroscopy, the NMR self-diffusion coefficient, and the proton longitudinal relaxation time of hydration water as a function of the temperature reveals a strong link between water dynamics and the two dynamical transitions of the protein. At high temperature, where the lysozyme denaturation process occurs, we observe that the population of the non-hydrogen-bonded fraction of water molecules is dominant. This latter result can be considered as a strong signal that changes in hydration water accompany the well known process of protein thermal unfolding. The low-temperature FSC dynamic crossover transition at about 225 K is related to the protein "glass" transition which, according to the recent neutron scattering data on the $\langle x^2 \rangle$,[25] is triggered by the strong coupling between protein and the hydration water. However, our experiments which focus on hydration water dynamics show unambiguously that both protein transitions are connected to the change of local hydrogen bond pattern of the hydration water which in turn leads to mobility changes of both the hydration water and the protein.

IV. METHODS

Hen egg white lysozyme used in this experiment was obtained from Fluka (L7651 three times crystallized, dialyzed, and lyophilized) and used without further purification. Samples were dried, hydrated isopiestically, and controlled by means of a precise procedure.[25] We used three samples with hydration levels $h=0.3$, 0.37, and 0.48. FTIR absorption measurements were performed at ambient pressure in the HOH bending and OHS spectral regions, using a Bomem DA8 Fourier transform spectrometer, operating with a Globar source, in combination with a KBr beamsplitter and a DTGS/KBr detector. To avoid saturation effects, we used the attenuated total reflection (ATR) geometry, generally insensitive to sample thickness. The spectra of interest were recorded with the resolution of 5 cm^{-1}, automatically adding 200 repetitive scans in order to obtain a good signal-to-noise ratio and highly reproducible spectra; then they are normalized by taking into account the effective number of absorbers.

The NMR dynamical properties of the hydration water were studied at ambient pressure and different temperatures using a Bruker Avance spectrometer, operating at 700 MHz ^{1}H resonance frequency. In these NMR experiments, we have measured the self-diffusion coefficient of water D and the proton spin-lattice relaxation time constant (i.e., T_1). D was measured with the pulsed gradient spin-echo technique ^{1}H-PGSE.[35] For the measure of T_1 we used the standard inversion recovery pulse sequence ($[\pi\text{-}t\text{-}\pi/2$ acquisition], where t denotes the time between the two rf pulses). In contrast to the case of pure bulk water for which the obtained spectra are represented by only one exponential time decay, the T_1 of the protein hydration water spectra can be fitted only by considering two exponential contributions; one due to the surface water and the second one to the protein protons.

The analysis of the measured OHS spectra is done considering the spectral contributions of two classes of oscillators (HB and NHB).[32] For bulk liquid water, in the range $283 < T < 368$ K, these OHS oscillators are described, as observed by Raman scattering and infrared absorption, by the following Gaussian peak positions (wave number):[32] (I) 3220 cm^{-1}, (IIa) 3400 cm^{-1}, (IIb) 3540 cm^{-1}, and (IIc) 3620 cm^{-1} (all with an experimental error of ±20 cm^{-1}). By means of proper considerations on the mean molecular polarizability α and the anisotropy β, HB oscillators have been assigned to the contribution I (the lowest in wave number), i.e., to bounded water molecules having a coordination number close to four, those able to generate HB networks. Conversely, the highest wave-number components IIb and IIc are ascribed to molecules poorly connected to their environment

and to multimer molecules, respectively. Between these Gaussians lies the component IIa, associated with water molecules having an average degree of connection lower than those coordinated in tetrahedral structures. All the experiments on water have confirmed this model, in particular, the thermal evolution of OHS spectra indicates that when T increases the HB OHS oscillators are replaced by the NHB ones at a higher frequency (vice versa on decreasing T). However, this picture is exact for $T \geqslant 283$ K. The exploration of the OHS spectra in the supercooled regime down to 246 K has indicated the presence of another Gaussian contribution (III), that arises at about 284 K and centered at about 3120 cm^{-1}, whose magnitude increases as T decreases.[36] Very recently, by studying confined water in the range 190 K $<T<$ 290 K we discovered that such a contribution dominates the OHS spectra below the FSC temperature T_L. Comparison with the LDA spectra allows us to identify such contribution with that of the LDL phase.

A first comparison of the OHS-FTIR spectra for the protein hydration water [Fig. 1(a)], reveals significant variations, on changing T, in the HB and NHB populations and the presence of this latter spectral contribution (III) in the supercooled regime. On the basis of the above considerations and the suggestions from the scenario portrayed in Fig. 1(a), we have studied the measured OHS spectra by means of a spectral deconvolution with three Gaussian components (Fig. 2). The first one is the III (3100 cm^{-1}) contribution assigned to the LDL phase, the second one is represented by the component I (3220 cm^{-1}), and finally a third Gaussian (at the highest frequencies) is related to the contribution of the NHB (or weakly HB) components. The spectral deconvolution was done by using a best fit procedure.

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