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Communication: Protein dynamical transition vs. liquid-liquid phase transition in protein hydration water

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In this work, we compare experimental data on myoglobin hydrated powders from elastic neutron scattering, broadband dielectric spectroscopy, and differential scanning calorimetry. Our aim is to obtain new insights on the connection between the protein dynamical transition, a fundamental phenomenon observed in proteins whose physical origin is highly debated, and the liquid-liquid phase transition (LLPT) possibly occurring in protein hydration water and related to the existence of a low temperature critical point in supercooled water. Our results provide a consistent thermodynamic/dynamic description which gives experimental support to the LLPT hypothesis and further reveals how fundamental properties of water and proteins are tightly related. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4822250]

Protein dynamics is characterized by anharmonic atomic fluctuations, which are essential for protein functioning. The main activation of this kind of motions in hydrated proteins is the well-known "protein dynamical transition" (PDT), a steep increase in the amplitude of atomic motions revealed at about 220–240 K by different techniques like neutron scattering.[1] The physical origin of this transition is still unclear, and different models have been proposed to explain it.[2–10] One of these proposes a connection of PDT with the existence of a low-temperature critical point[11] separating two distinct forms of supercooled liquid water (like protein hydration water), a low density liquid (LDL), and a high density liquid (HDL). In this scenario, originally proposed by Chen and co-workers,[3,12–15] interfacial water on protein surface undergoes a first order transition at 220–230 K (Liquid-Liquid Phase Transition, LLPT), where the LDL→HDL structural transition is connected with a change in water dynamical properties revealed as a crossover from an Arrhenius to a super-Arrhenius temperature dependence of the relaxation times. The LLPT hypothesis for supercooled water has been supported also by computational studies,[16–18] but recently questioned by Limmer and Chandler.[19,20] A model based on the cooperativity of a 2-dimensional hydrogen network has been also proposed, where two dynamic crossovers are expected to occur at 180 and 250 K (at ambient pressure), the latter consistent with the presence of a low-temperature water critical point.[21] Other experimental works investigating the temperature dependence of water relaxation times have seriously questioned the existence of the LLPT in the protein hydration water.[22–24]

If the LLPT actually occurs, the coupling between hydration water and protein molecular groups would cause, in turn, the activation of anharmonic protein motions, revealed by neutron scattering as an enhancement of protein mean square fluctuations and identified as the PDT. Further support to this picture of the PDT has been given by a recent neutron scattering work on homomeric polypeptides,[8,25] where the dependence of PDT onset temperature and fluctuations amplitude on energy resolution has been used to investigate the energetic details of this phenomenon. Here, we look for further experimental evidence of the connection between PDT and hydration water LLPT by using a combination of different experimental techniques sensitive to hydration water and protein, on the very same system. We report dynamic and thermodynamic experimental information on myoglobin (Mb) D_2O-hydrated powders from elastic neutron scattering (ENS), broadband dielectric spectroscopy (BDS), and differential scanning calorimetry (DSC). Two different hydration levels are investigated: h = 0.3 [gr D_2O]/[gr Mb] (corresponding approximately to a single water layer on the protein surface[26]) and h = 0.5 [gr D_2O]/[gr Mb] (where a number of water molecules do not interact directly with the protein surface). Samples have been prepared as described in Ref. 27.

ENS temperature scans were performed at the thermal backscattering spectrometer IN13 (Institut Laue-Langevin, Grenoble, France) with an incident wavelength $\lambda = 2.23$ Å and an energy resolution of 8 μeV FWHM. Mean square displacements (MSDs) of non-exchangeable H atoms of Mb were obtained from neutron scattering function as described in Ref. 28. Briefly, according to the Gaussian approximation,[29] we used the following definition:

$$\frac{S(Q,T,E=0)}{S(Q,T=20\,\mathrm{K},E=0)} = \exp\left[-\frac{\langle u^2\rangle(T)-\langle u^2\rangle(20\,\mathrm{K})}{6}Q^2\right] = \exp\left[-\frac{\langle \Delta u^2\rangle(T)}{6}Q^2\right],$$

where $S(Q,T,E)$ is the dynamic structure factor, i.e., the neutron scattering intensity as a function of momentum transfer (Q), temperature (T), and exchanged energy (E); $\langle u^2\rangle$ is the total mean square displacement of protein hydrogen

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atoms. MSD is defined as $\langle \Delta u^2 \rangle/6$. The quantity ΔMSD(T) = [MSD(T)$_{hydrated}$ − MSD(T)$_{dry}$]/[MSD(298 K)$_{hydrated}$ − MSD(298 K)$_{dry}$] was calculated subtracting the MSDs relative to a Mb dry sample (where PDT does not occur) from those relative to the hydrated ones, and then normalizing for the difference calculated at room temperature.[8] This procedure allows to subtract contributions related to the methyl groups activation (that are hydration independent[30]) and to normalize for the resolution dependent MSD amplitude. BDS measurements were performed in the frequency range 10^{-2}–10^{7} Hz using a Novocontrol Alpha analyzer. Sample diameter and thickness were 28 and 1 mm, respectively. As described in more detail in Ref. 27, dielectric spectra, namely, the complex permittivity ε^* vs. frequency ν, were fitted (real and imaginary parts simultaneously) by a combination of Havriliak-Negami functions, a term for direct conductivity σ and an additional term to take into account effects of electrode and interface polarization:

$$\varepsilon^* = \varepsilon_\infty + \sum_j \frac{\Delta\varepsilon_j}{\left[1 + \left(i2\pi\nu\tau_j\right)^{\alpha_j}\right]^{\beta_j}} + i\frac{\sigma}{2\pi\nu} + (a + ib)\,\nu^{-\lambda},$$

where ε_∞ is the high frequency limit of permittivity, $\Delta\varepsilon_j$ is the dielectric strength, τ_j is the relaxation time of the jth relaxation process, and λ is a parameter describing the fractal character of polarization processes. DSC measurements were performed with a Pyris Diamond (Perkin-Elmer) calorimeter. Samples of Mb hydrated powders were sealed in steel pans of ~60 μl and cooled to 90 K with a cooling rate of 20 K/min; calorimetric upscans from 90 to 300 K were then performed with a heating rate of 20 K/min.

In Figure 1, we show the results obtained using the above described experimental techniques on the Mb protein powders in the temperature range 100–300 K. A thermodynamic description of the system is achieved by DSC (panel (a)). The absence of any transition in the dry (h = 0) sample indicates that the features observed in the hydrated ones refer to water. In particular, hydration water exhibits a glass transition at about 170 K,[24] where glassy water becomes liquid, and a first-order liquid-liquid transition starting at about 230 K, analogously to what has been observed in supercooled water confined in Vycor porous matrices, where neutron diffraction data identified this first-order transition as the LDL→HDL structural change, since it correlated with a change in the water intermolecular distance.[31] An additional endothermic peak is observed at higher temperature (T_{onset} ~ 280 K) in the sample at h = 0.5, compatible with the melting point of heavy water and indicating that water molecules out of the first hydration shell may form ice, as already reported.[32] Panels (b) and (c) report the relaxation times measured by BDS. As already discussed in Ref. 27, three main relaxation processes are observed: a fast relaxation (FR), highly dependent in both amplitude and relaxation time on the hydration level, attributed to the collective relaxation of hydration water; an intermediate relaxation (IR), showing an Arrhenius-superArrhenius crossover at about 230 K and nearly independent of hydration level, attributed to the water molecules strongly interacting with protein surface; a slow relaxation (SR) attributed in Ref. 27 to motions of charged/polar groups in the protein interior. Interestingly, as evidenced by the arrows in Figures 1(b) and 1(c), the characteristic time of the FR reaches 100 s (which is a common dynamical definition of the glass transition) at a temperature compatible with the glass transition onset temperature detected by DSC; this clearly links the FR to the viscosity-related relaxation of the hydration water. On the other hand, the crossover observed in the temperature dependence of the IR occurs at the same temperature of the LLPT onset observed in the calorimetric scans. The normalized ΔMSDs of non-exchangeable H atoms of Mb are reported in panel (d), where the typical kink in the MSD temperature dependence corresponding to the PDT is clearly observed. As evidenced by the yellow area in Figure 1, the PDT occurs exactly in the same temperature region of the liquid-liquid thermodynamic/dynamic transition of hydration water.

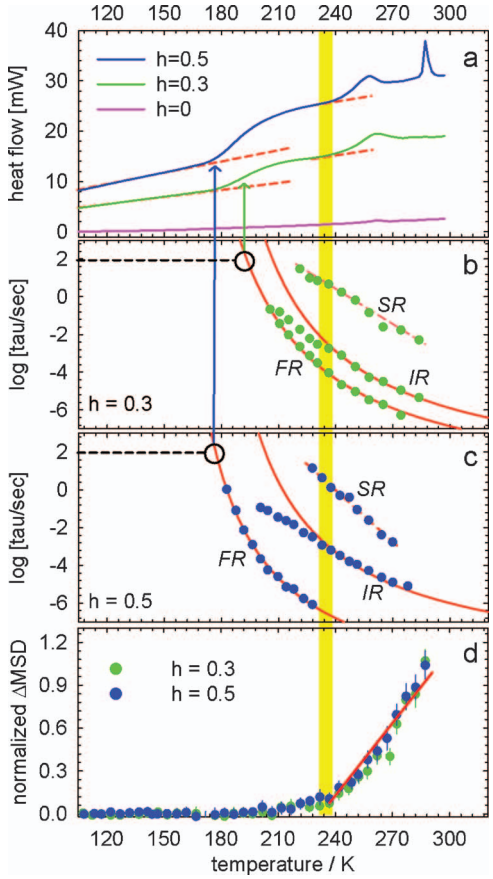

FIG. 1. Experimental data obtained on D_2O-hydrated Mb powders. From top to bottom: (a) differential scanning calorimetry up-scans; the accuracy of heat flow and temperature estimation is <±1% and ±0.1 °C, respectively; (b) and (c) relaxation times obtained by broadband dielectric spectroscopy; error bars are not shown since they are of the order (or less) of the points dimension; (d) mean square displacements of non-exchangeable H atoms of Mb obtained by elastic neutron scattering. Yellow area indicates the temperature region where hydration water shows a first-order liquid-liquid transition (panel (a)) and an Arrhenius-superArrhenius crossover (panels (b) and (c)), while non-exchangeable H atoms of Mb undergo the PDT (panel (d)). The red line in panel (d) is a guide to the eye.

The combined use of the different experimental techniques reported here furnishes a coherent description of the PDT. Protein hydration water, after exhibiting a glass transition at about 170 K, experiences a first-order liquid-liquid

transition at about 230 K, that we can identify with the LDL→HDL crossover observed in supercooled water, in analogy with what observed in other supercooled interfacial water systems.[31] This thermodynamic event has a dynamical counterpart in a change of cooperativity of the relevant water relaxation process, as revealed by dielectric measurements. As already reported,[11] this change is related to a crossover from an almost fully tetrahedrally H-bonded, "strong" LDL to a "fragile" HDL, whose structure and dynamics are similar to that of supercooled bulk water at higher temperature. Water molecules involved in the crossover (i.e., interfacial water molecules) are tightly coupled with protein molecular groups on protein surface via hydrogen bonds and electrostatic interactions,[33] and induce an increase of protein atomic fluctuations, which is revealed by neutron scattering as the PDT. This interpretation is also compatible with the absence of PDT in dry proteins, as experimentally proven.

In conclusion, the results presented here give further experimental evidence that a fundamental property of hydrated protein molecules is associated to a phase transition in hydration water, which is supposed to be a general phenomenon occurring in supercooled water.

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