Hydration water rotational motion as a source of configurational entropy driving protein dynamics. Crossovers at 150 and 220 K

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The existence of a protein dynamic transition around 220 K is widely known and the central role of the protein hydration shell is now largely recognized as the driving force for this transition. In this paper, we propose a mechanism, at the molecular level, for the contribution of hydration water. In particular, we identify the key importance of rotational motion of the hydration water as a source of configurational entropy triggering (i) the 220 K protein dynamic crossover (the so-called dynamic transition) but also (ii) a much less intense and scarcely reported protein dynamic crossover, associated to a calorimetric glass transition, at 150 K.

I. Introduction

The sudden change of slope in the temperature dependence of hydrated proteins mean-square displacement around 220 K has been extensively studied (for a review see ref. 1 and references therein). The reason for such focusing on this so-called dynamic transition is twofold. First, this transition is intimately connected to the protein function. Then, and this is even more intriguing, this connection can be made for a wide variety of systems from small soluble globular proteins to membrane proteins. In the early steps the role of the solvent surrounding the proteins has been recognized: in the absence of hydration the 220 K dynamic transition vanishes. Also, the transition temperature is controlled by the viscosity of the solvent: the transition temperature is raised in the presence of co-solvents like sugar. A consensus has now emerged over this idea of a deep interplay of the proteins motions, and therefore function, with the surrounding solvent.

Dynamic processes at play in water physics are now well understood, at least in bulk water. In bulk, a water molecule is hydrogen bonded, on average, to slightly less than four neighbouring molecules. If due to thermal energy, a hydrogen bond O···H–O moves apart from linearity by an angle larger than 25°, the bond breaks. When several H-bonds engaged by a molecule are simultaneously broken, the molecule is free to experience a rotational diffusive motion until several hydrogen bonds are formed, again leading to the formation of a transient localization "site". It is a key point to note that within this mechanism, long range translational dynamics can only occur if rotational dynamics is present. In other words, translation is driven by rotations. It therefore clearly appears that the notion of "water dynamics" is a convenient shortcut but is actually rather vague and imprecise. Getting a real insight in the physics of water or hydration related phenomena, requires to distinguish rotational and translational contributions.

In interfacial water, when dealing with just a monolayer of water molecules, the mechanism above is still at play with an average number of H-bonds per molecule reduced to three.[2] In a previous study,[3] we have been able to discriminate between the rotational and translational contributions of water molecules in such a monolayer situation at a hydrophilic surface. The originality of this work was not only to discriminate between the translational and rotational water components but also to perform this decomposition over a wide range of temperature from 70 to 280 K. The present paper extends these results, to the very active field of biophysics devoted to understanding how the function of a bio-molecule can be shaped by the structural and dynamic properties of the surrounding solvent.

II. Materials and methods

A Interfacial water

In this paper we refer to interfacial water samples as water adsorbed as a monolayer on Vycor,[4] a porous silica glass. The surface of Vycor is covered with silanol groups (Si–OH) so that water molecules specifically interact both with the surface and between them through hydrogen bonds. A partially hydrated sample was prepared by absorption of water in the vapor phase, until a level of hydration of 0.06 g H_2O g^{-1} corresponding to monolayer coverage was obtained.

B Hydrated lysozyme

Salt-free lyophilized hen egg white lysozyme (14.3 kDa) (Sigma company) was first dissolved in pure D_2O at a concentration of 5 mM, then extensively dialyzed against D_2O, to exchange labile protons. The sample was then lyophilized. The hydrated powder was obtained by rehydrating the lyophilized material by vapour phase adsorption of D_2O. The water content of the samples was determined by weighing. The hydrated powder samples containing 300 mg of dry protein,

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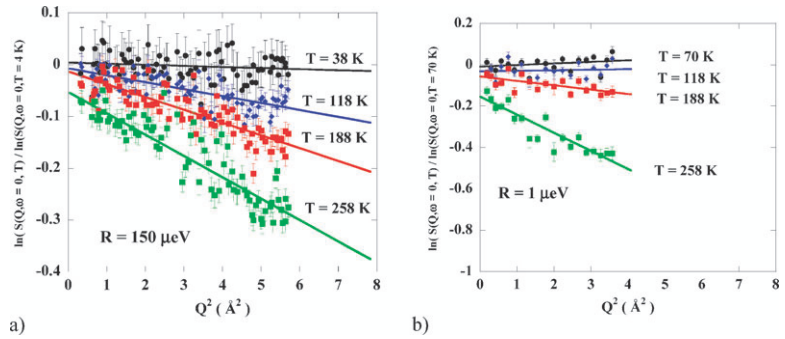

Fig. 1 $\mathrm{Ln}(S_{protein}(Q,\omega \approx 0))$ *vs.* Q^2 plots and the corresponding linear fit for selected temperatures for (a) $R = 150$ μeV and (b) $R = 1$ μeV data. In each case, the data have been normalized to the elastic intensity of the lowest temperature measured.

was sealed in a thin-walled aluminium cell. The level of hydration was 0.4 g D_2O g^{-1}.

C Neutron scattering

The high energy resolution experiment, assessing dynamics up to 1 nanosecond, was performed on the IN16 spectrometer (ILL, Grenoble, France) (6.28 Å incident wavelength, energy resolution 1 μeV, dynamic range from ±12 μeV and a Q range of 0.1 to 1.9 $Å^{-1}$). Lower energy resolution data, adapted for correlation times up to 10 ps, were measured using 5.0 Å incident neutrons, over a broad dynamic range −2 to 150 meV using a 150 μeV low resolution/high flux mode of the Mibémol spectrometer (LLB/ Saclay) over the Q range 0.3 to 2.0 $Å^{-1}$. Complementary experiments, at an intermediate resolution (80 μeV) and a Q range 0.3 $Å^{-1}$ to 2.5 $Å^{-1}$ were performed at QENS, ANL/IPNS.

III. Results

Owing to the large incoherent neutron scattering cross-section of the ^1H nucleus and the abundance of this element in proteins, incoherent inelastic neutron scattering measurements are able to give a global view of protein dynamics as sensed *via* the averaged individual motions of its hydrogen atoms. Due to the strong difference of the neutron scattering cross-sections of hydrogen ($\sigma_{inc}(H) = 80.26$ barn) and deuterium ($\sigma_{inc}(D) = 2.05$ barn) the contribution of the deuterated hydration water is negligible.

In general, the dynamics probed by inelastic neutron spectroscopy will depend on the scattering vector, Q, and the energy range, ω, accessible by a specific neutron spectrometer. It should be noted that all the results presented in the present paper are derived from elastic scans. This is a particular way of conducting the neutron experiment by imposing a weak but constant temperature slope (typically 20 K h^{-1}) to the sample and concurrently continuously recording for a few minutes the Q dependence of the elastic neutron intensity. For purely vibrational systems, in a way similar to the X-ray Debye–Waller effect, at each temperature the Q dependent Gaussian intensity loss $\exp(-Q^2 \cdot \langle u^2 \rangle/3.)$ can be related to the spatial extension $\langle u^2 \rangle$ of atoms, here mainly protons, around their equilibrium position. No neutron energy loss or gain is recorded so that no direct inelastic information (*i.e.* correlation times) can be derived from such experiments. Instead, the sample dynamics is indirectly probed by evaluating protons mean-square displacement (MSD). It should be noted that the high Q elastic intensity loss is strictly speaking Gaussian, only in the case of purely harmonic vibrational systems. As a matter of fact, the 220 K overshoot of the protein MSD is due to an additive relaxational contribution (anharmonic, diffusive-like and/or over-damped motion, for example).

Elastic scans of the 0.4 g D_2O g^{-1} hydrated lysozyme have been measured from 4 to 300 K, on Mibémol ($R = 150$ μeV, Fig. 1a), and from 70 to 280 K on IN16 ($R = 1$ μeV, Fig. 1b). As shown in Fig. 1a and b, at all temperatures, a single line was sufficient for a satisfactory fit of the $\ln(S_{protein}(Q,\omega \approx 0))$ *vs.* Q^2 plot over the entire Q range. The corresponding temperature dependence of the lysozyme MSD, at 150 μeV is shown in Fig. 2a and b. The dynamic transition is clearly detected at 220 K. As shown in Fig. 2b, another transition is

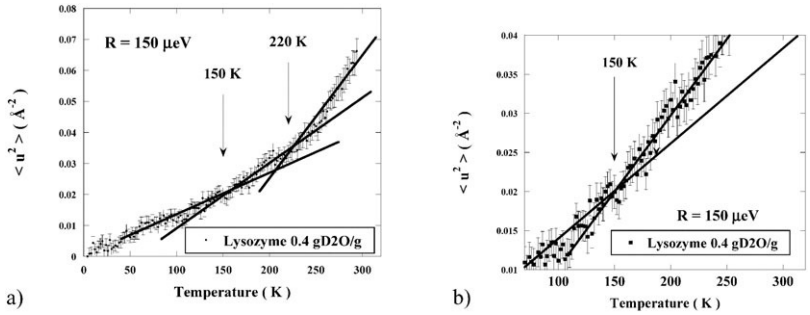

Fig. 2 (a) MSD of 0.4 g D_2O g^{-1} hydrated lysozyme. At the low resolution used here (150 μeV) the protein proton dynamics is probed on a timescale extending up to 10 ps. Even on such a short timescale, the 220 K dynamic transition is clearly detected. A much more subtle transition is also detected at 150 K. (b) Same as (a) but focusing on the low temperature region to stress out the 150 K transition.

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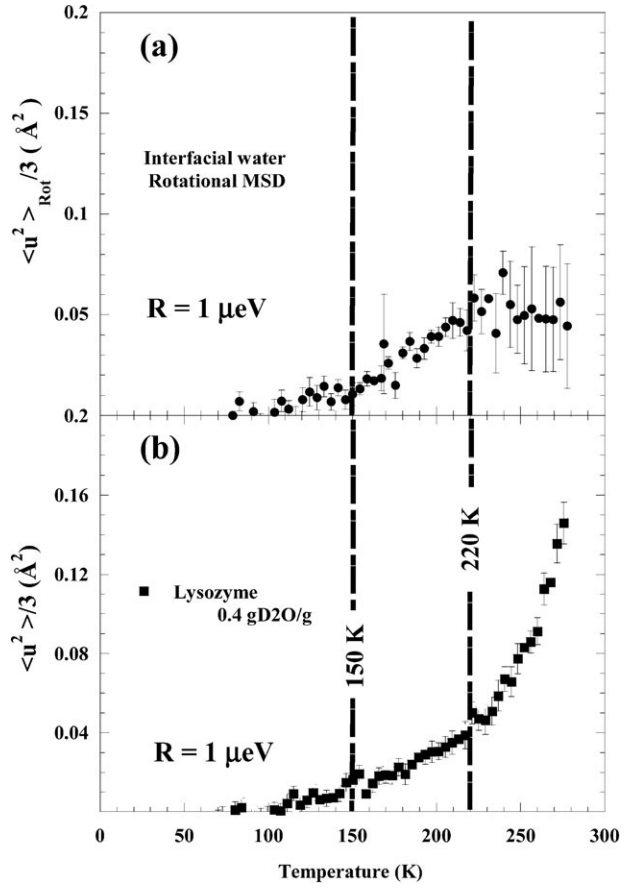

Fig. 3 (a) Temperature dependence of (a) the interfacial water rotational mean-square displacement (MSD) and (b) dynamic transition in 0.4 $gD_2O\ g^{-1}$ hydrated lysozyme. The instrumental conditions were strictly identical for both experiments. At the 1 μeV resolution used presently (IN16, ILL), motions are probed up to a few ns. A correlation is shown between the onset of interfacial water rotational motion at 150 K and a transition in lysozyme protons MSD. This correlation is also clearly detected at 220 K. As shown in ref. 3, above 220 K the water rotational correlation time suddenly decreases and within the statistics, the related quasi-elastic signal manifests itself, on the narrow IN16 dynamic range, as a temperature independent background. The plateauing of the water MSD above 220 K is therefore only apparent and due to instrumental effects (see Fig. 4). Such a MSD plateau is not seen in the case of the protein because a significant fraction of the large distribution of the protein correlation times lies in the nanosecond time-range. Data in (a) from ref. 3.

also detected at 150 K. The same behaviour of lysozyme MSD is obtained at 1 μeV (see Fig. 3b).

Elastic scans have been measured on interfacial water samples using the backscattering IN16 spectrometer (ILL, France). We have shown in a previous paper[3] that two Q regions had to be defined in order to properly take into account the Q dependence of the elastic intensity loss. The two distinct MSD derived from the small ([0.1–1.0 $Å^{-1}$]) and the high Q ([1.1–1.9 $Å^{-1}$]) regions were, respectively, attributed to long range translational, $\langle u^2\rangle_{trans}$, and local rotational, $\langle u^2\rangle_{rot}$, MSD contributions to water dynamics. The $\langle u^2\rangle_{rot}$ contribution, we are focusing on in this paper is reported in Fig. 3a.

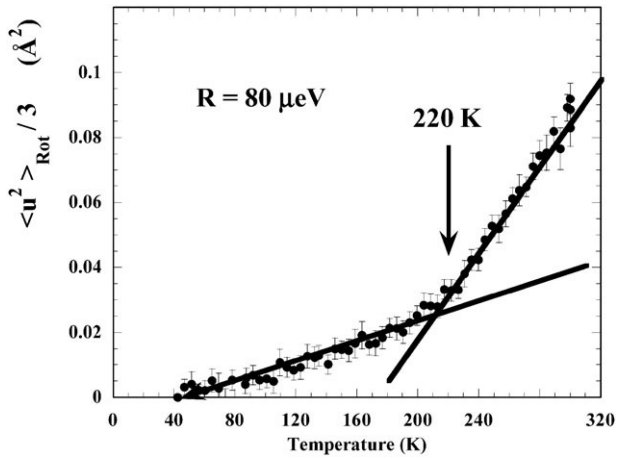

Fig. 4 Temperature dependence of the interfacial water rotational mean-square displacement (MSD) at 80 μeV energy resolution (QENS, ANL/IPNS). At this resolution, the 220 K water rotational motion MSD is fully detected. Data from ref. 3.

We have shown that (for details see ref. 3):

(i) $\langle u^2\rangle_{rot}$ significantly increases above 150 K. This is the signature of the onset of rotational dynamics of the water molecules.

(ii) Below 165 K, $\langle u^2\rangle_{Trans} \approx 0$ *i.e.* no interfacial water long range translational dynamics is detected. Above 165 K, no change is observed in water density (not shown) but translational dynamics becomes possible on a 1 ns timescale, as shown by the $\langle u^2\rangle_{Trans}$ non-null value. This is a glass transition in interfacial water at 165 K.

(iii) Above 220 K, rotational and translational correlation times related to $\langle u^2\rangle_{trans}$ and $\langle u^2\rangle_{rot}$ significantly decrease, becoming too short to be measured on the IN16 narrow energy window (±12 μeV). The flattening out of the measured MSD, shown on Fig. 3a above 220 K, is therefore not physical but due to an instrumental limitation. This instrumental limitation is overcome by an experiment at lower resolution (80 μeV) in a broader dynamic range (several meV) as shown in Fig. 4.

IV. Discussion

It is now widely accepted that hydration plays a crucial role in the connection between the dynamics of biological molecules and their function.[5] In a previous paper we have tackled[6] (using concurrent ^{13}C solid state NMR and quasi-elastic neutron scattering (QENS)) this issue of the influence of hydration on protein dynamics, in the case of parvalbumin, a small (11.5 kDa) soluble globular protein. The hydration has been varied from the dry state (lyophilized powder) up to 0.65 $g\ g^{-1}$ and the experiment was performed at room temperature. The combined NMR (ns timescale) and QENS (15 ps timescale) results suggested that peripheral water–protein interactions influence the protein dynamics in a global manner, with a progressive induction of mobility, at increasing hydration, from the periphery toward the protein interior. This interpretation has been shown to be consistent with subsequent molecular dynamics (MD) simulation results by Hinsen *et al.*[7] For each atom of the protein, these authors estimate a

local density and define a local friction coefficient. They show a convincing linear increase of the friction coefficient *vs.* the local density, suggesting that the core of the protein experiences larger friction, *i.e.* longer diffusive-like correlation times responsible for the QENS signal, than the atoms at the protein surface. At a second level, this distribution of friction coefficients is a direct evidence of the large dynamical heterogeneity (large distribution of correlation times) in proteins.

We therefore end up with the following image, valid at room temperature, of a constrained protein dynamics at the core of a globular protein surrounded by fluid-like dynamics at its surface, surface which is in close contact with the hydration water. If the central role of hydration water in triggering protein dynamics is widely accepted, very few attempts have been made to elucidate at the molecular level the detailed mechanism by which water molecules drive, at least partly, the protein motions. Tarek and Tobias[8] suggest that water translational motion is necessary for the structural relaxation that permits anharmonic and diffusive motions in proteins. Furthermore, it appears that the exchange of protein–water hydrogen bonds by water rotational/librational motion is not sufficient to permit protein structural relaxation.

In this paper, we use Vycor, a hydrophilic glass, to mimic the hydrophilic surface of a globular protein. The surface of Vycor, covered by a homogeneous layer of silanol (Si–OH) groups, is obviously quite a crude model of a protein surface where hydrophilic, hydrophobic, polar or even charged groups are in constant direct or indirect dynamical interaction. However the use of this simple, but well defined, model system offers the strong advantages of (i) being stable in a broad temperature range and (ii) being dynamically inert: it has no dynamical contribution in the time (ps up to ns) and the temperature window of 4–300 K under scrutiny in this paper. We can therefore probe the pure and intrinsic dynamic behaviour of a water monolayer in interaction with a hydrophilic surface in the 4–300 K broad temperature range.

The study of a biological system at temperature as low as 4 K, as in the present paper, has at first sight little direct biological relevance. Tracking the dynamical response of the system over such a broad temperature range is, nevertheless, generally an excellent methodological way to discriminate between the dynamic modes of different energies at play in a complex system. This is shown here in the case of interfacial water where full analysis of the Q dependence of the elastic intensity over a broad temperature range has led to a successful separation of translational and rotational contributions to the interfacial water MSD.

It should nevertheless be noted that MSD values are not direct dynamical quantities since they just provide an estimate of the extension induced by a mode with no information on its characteristic time. Time dependent quantities, like diffusion coefficients, residence times or vibrational correlation times can be obtained by a full analysis of the energy dependence of the neutron scattered intensity, $S(Q,\omega)$. However, such analysis is somehow made difficult since, depending of its specific time resolution, an inelastic neutron spectrometer provides access only to correlation times lying in a specific time window. An example is given here in the case of the rotational motion of interfacial water: on IN16 (1 ns time-scale) up to 220 K, $S(Q,\omega)$ shows, a very little and very narrow quasi-elastic signal. Then, in a narrow temperature range above 220 K, the water rotational correlation time decreases suddenly, giving rise to a broad quasi-elastic signal detected as a flat background in the IN16 narrow dynamic range. Then, above 220 K the dynamic information is in some way too short for the time window of IN16; it manifests itself as a flat background which, within the statistics, is almost temperature independent. As a result $\langle u^2 \rangle_{rot}$ shows a temperature independent plateau (Fig. 3a). Instead, at lower resolution (Fig. 4), the sudden change in the water rotational dynamics is clearly detected above 220 K, and can be followed up to room temperature where a significant quasi-elastic signal is detected. In any case on data shown here, below 220 K, no water quasi-elastic signal is detected, thus no line-shape analysis in energy is possible and no rotational diffusion coefficient can be extracted from the data below that temperature. Experimental techniques accessing correlation times longer than a nanosecond, like NMR, can then be benefited from. Nevertheless, as shown here in the case of interfacial water, probing both the time (*i.e.* ω) and spatial (*i.e.* Q) dependence of the dynamic observables is a key point to discriminating between different dynamics (here translation and rotation). So, elastic scans, even if they are frustrating since they do not provide any direct dynamic information, are nevertheless informative. In the present case, where neutron spin-echo is impracticable due to the low signal to noise ratio of a water monolayer on Vycor, even if they do it in an indirect way (MSD instead of correlation times) elastic scans are even the only way-through to get insight in the low temperature (long time) dynamics of interfacial water. A caveat nevertheless exists in the excessive use of elastic scans to probe the dynamics of a system. Protein dynamics is actually a good example where elastic scan-derived information can be misleading. Above the zero-point energy motion and below the 220 K MSD onset, $\langle u^2 \rangle$ shows a linear dependence over temperature. This is evidence that protein protons experience purely high (few tens of meV) energy harmonic vibrational dynamics with no damping. Above 220 K, this temperature linear regime ends. This is the signature of new lower energy dynamic modes like overdamped or anharmonic vibrations. In our view, it is a key point to realize that above 220 K, since the system becomes highly dynamically heterogeneous (see discussion above), it cannot be characterized by a simple single quantity like, for example, resilience.[9]

The temperature dependence of the protein proton MSD is shown in Fig. 3b. We recall that we are dealing with a hydrated protein powder sample. In such a system, on a timescale extending only to a few nanoseconds, the centre of mass of a specific protein is fixed in space. On average, the motion of the protein protons (actually mainly side-chain protons) is a complex trajectory resulting in successive dihedral reorientations. We have shown earlier,[10] that such a trajectory can be accounted for by a diffusion inside a sphere with a radius of a few angströms. Therefore in the conditions we are dealing with in this paper, protein motions do not experience any long range motions. This is, in our view, why a single line is sufficient to fit the $\mathrm{Ln}(S_{protein}(Q,\omega \approx 0))$ *vs.* Q^2 curves. As a consequence, we are establishing a connection

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between the interfacial water rotational motion, $\langle u^2 \rangle_{rot}$ and the protein motion $\langle u^2 \rangle_{protein}$.

As shown in Fig. 3b, the 220 K protein dynamic transition is connected to a transition at the same temperature in the rotational behaviour of interfacial water. Recently, such a 220 K transition of protein hydration water has been detected by neutron scattering. It has been proposed that at this temperature, water experiences a fragile-to-strong dynamic crossover (FSC) as a result of a structural transition of hydration water from a high-density to a low density form.

The transition of hydration water at 220 K is the object of hot debates. Recently, based on dielectric spectroscopy studies of hydrated lysozyme, Pawlus *et al.*[11] did not detect any transition but instead showed a smooth behaviour of the whole system (protein and hydration water) over a temperature range [173–253 K]. These authors therefore dismiss any fragile-to-strong crossover in the hydration water of protein. They suggest that the sharp rise in protein MSD clearly observed in neutron scattering experiments is indirectly an instrumental effect arising from the fact that at 220 K, the relaxation times of the system match the time window of the spectrometer.

As a matter of fact, such resolution effects can have serious consequences on dynamic observables[1] or indirect effects on the related MSD (see Fig. 3a with the $\langle u^2 \rangle_{rot}$ flattening out above 220 K and the Fig. 4 where the transition is clearly detected at lower resolution). Nevertheless, Chen *et al.*[12] have measured by NMR a clear anomaly at 223 K in the diffusion coefficient of hydration water in 0.3 g g^{-1} hydrated lysozyme.

The data lying in the 220 K region presented in the present paper do not bring any additional information connected to the existence of a FSC of protein hydration water at 220 K. A similar singular point of interfacial water behaviour (water monolayer on Vycor) had been proposed earlier[3] and on the basis of diffraction and calorimetric results it was identified to happen at 240 K. This liquid–liquid transition has important consequences in explaining the anomalies of water in conjunction with the existence of a low temperature critical point in bulk water.[13] However, since the Vycor can be considered as a dynamically inert material in the temperature range of interest, a very important input of the data presented here is the fact that interaction of water with a protein (which is not dynamically inert) is not needed to drive a dynamic transition in interfacial water. We think that the correlation between water and protein dynamics can be put a step further and in our view, the driving force of the protein dynamics is hydration water. It is likely, however, that as a second order effect, the dynamics and the structure of the protein surface influence in turn the interfacial water dynamics. Such an effect is probably challenging to probe by experimental techniques, even by QENS with fully deuterated proteins[14,15] and light water) but could be studied by MD simulations.

We think that this conclusion can also be extended to the transition observed at 150 K. As shown in Fig. 3a. $\langle u^2 \rangle_{rot}$ significantly increases above 150 K. We have also shown by deep inelastic neutron scattering[16] that the hydrogen bond strength experiences a transition to become softer when the temperature is increased above the 150 K region. Our interpretation is that this is the temperature where the thermal energy is sufficient to allow significant excursion of the O···HO bond away from linearity, and as a consequence some hydrogen bonds can break and the configurational entropy of the system suddenly increases.

The strong correlation shown in Fig. 3 of the present paper strongly suggests that this transition in the interfacial water rotational dynamics is intimately connected to the protein transition at 150 K. To our knowledge, such a 150 K dynamic transition in hydrated proteins has only been scarcely reported.[17] Taking advantage of data collected at two very different energy resolutions, we show here that it is actually detected in two different time windows: ps but also ns.

The use of the word transition is widely used in the literature to describe the onset of MSD. It should be noted however that *stricto sensu*, a transition should be associated to a thermal event as detected by calorimetry. To our knowledge, this is not the case for the so-called 220 K dynamic transition.[18] This is why we prefer the term of dynamic crossover. As a matter of fact, a strong transition is detected[19] at 150 K in the specific heat of hydrated lysozyme crystals. This later phenomenon is the more direct evidence of a direct change in entropy fluctuations of the protein-water system at 150 K. Nevertheless, by calorimetry alone, it is not possible to discriminate if the transition is due to the protein, hydration water or both. Neutron scattering results shown here suggest that both the hydration water and the protein are experiencing a transition. A firm conclusion could be reached in following the MSD temperature dependence on a fully deuterated protein[14,15] sample hydrated by H_2O.

We would like to note that this 150 K transition of hydration water has a very practical consequence in a field of prime importance to biology: protein crystallography. Weik *et al.*[20] have shown that flash-cooled protein crystals are arrested in a metastable state up to 155 K without crystallization of the solvent. This is to be considered as a high temperature limit for the storage and handling of flash-cooled protein crystals.

V. Conclusion

We have shown that interfacial water at the surface of Vycor, a hydrophilic inert (chemically and dynamically) material, experiences different dynamic crossovers. As far as the rotational motion of water is concerned, transitions are detected at 150 and 220 K. At 150 K, the hydrogen-bond becomes softer.[16] But no change in the hydrogen-bond strength has been detected at 220 K. The 220 K dynamic crossover could then be associated to a structural change in hydrogen-bond connectivity.

Above 220 K, the interfacial water rotational motion lies within a time range between a few picoseconds up to a few tens of picoseconds time range (see plateauing in Fig. 3a and see Fig. 4), while due to a broad distribution of protein correlation times, protein dynamics is detected at least up to a nanosecond (Fig. 3b).

We have shown a strong parallel evolution at 150 and 220 K between the mean-square displacements related (i) to interfacial water rotational dynamics and (ii) to proton dynamics of a hydrated protein. This connection is made at the local scale (few angströms) and in the timescale of ns. We interpret

these observations as evidences that interfacial water rotational dynamics is the real source of entropy driving protein dynamics.

Altogether, we reach this final view of the protein-hydration water interaction and how this interaction can drive the protein function: the protein external side-chain short time motions, induced by fast water reorientational motion (leading here to $\langle u^2 \rangle_{\text{rot water}}$ Fig. 3a), propagate in a hierarchical way,[21] along the protein structure from the residue side chains down to the protein core to induce the longer timescale protein backbone motion necessary for protein function. The dynamic crossovers experienced by water at 150 and 220 K are also detected in the protein dynamics, even though the time scales of the crossovers can be different (longer times for protein than for interfacial water).

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