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Vibrational dynamics changes of protein hydration water across the dynamic transition

Alessandro Paciaroni [a,*], Andrea Orecchini [a], Federico Sebastiani [a,b], Simone Capaccioli [c,d], Kia L. Ngai [c,d], Martine Moulin [e], Michael Haertlein [e], Caterina Petrillo [a], Francesco Sacchetti [a,b]

[a] Dipartimento di Fisica e Geologia, Università degli Studi di Perugia, Via A. Pascoli, I-06123 Perugia, Italy
[b] CNR, Istituto Officina dei Materiali, Unità di Perugia, c/o Dipartimento di Fisica e Geologia, Università degli Studi di Perugia, I-06123 Perugia, Italy
[c] Dipartimento di Fisica, Università degli Studi di Pisa, Largo B. Pontecorvo 3, I-56127 Pisa, Italy
[d] CNR-IPCF, Institute for Chemical and Physical Processes, Largo B. Pontecorvo 3, I-56127 Pisa, Italy
[e] Deuteration Laboratory, Institut Laue-Langevin, 6 rue Horowitz, F-38042 Grenoble, France

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ABSTRACT

The vibrational dynamics of protein hydration water has been studied by incoherent neutron scattering. Experiments on a sample of fully deuterated maltose binding protein allowed us to single out the hydration water susceptibility. The main inelastic features, corresponding to hydrogen-bond bending, hydrogen-bond stretching and librational excitations, have been followed over a temperature range extending from 50 to 300 K. It turns out that the temperature dependence of the hydrogen-bond stretching contribution is quite similar to that of the mean square displacements deduced by the quasielastic signal, thus suggesting a close relationship between the anharmonicity of longitudinal phonon-like motions and the onset of diffusive molecular dynamics. On the other hand, both hydrogen-bond bending and librational excitations show a temperature dependence consistent with a harmonic character over the whole temperature range.

1. Introduction

Water molecules within a few Å of the protein surface are able to effectively perturb both the diffusive and vibrational dynamics of biomolecules, as shown by experimental [1–13] and theoretical [14–18] investigations. This kind of water, referred to as protein hydration water, has been shown to play a role in efficient enzymatic catalysis [19], in folding processes [20–22], as well as in molecular recognition and in mediating protein–protein interactions. Indeed, fluctuations in the hydration shell have been suggested to control fast fluctuations of proteins, thus having profound implications for their biological activity by driving key internal processes such as the migration of ligands within myoglobin [23].

Apart from its crucial biological role, protein hydration water is also a unique system to study the physical properties of the water molecular network in conditions where it is sensibly altered with respect to the bulk, due to local topography and specific interactions with the protein surface [24]. As for structural features, the average value of the mass density of protein hydration water has been shown to be definitely larger than that of bulk water by X-ray [25] and small angle neutron scattering [26] experiments, by molecular dynamics (MD) [27] and Monte Carlo [28] simulations. In addition, the density of protein hydration water can be locally lower or higher than the bulk, depending on the curvature of the protein surface [27]. Also dynamic properties of water molecules at the protein interface are quite different from the bulk, with a large fraction of them experiencing a moderate slowdown factor of ~2–3 [29,30]. This slowdown has been interpreted in terms of a theoretical extended jump model and ascribed to both a topological excluded-volume factor, resulting from the local protein geometry, and a free-energy factor, arising from the water–protein hydrogen-bond strength [29].

The temperature dependence of these diffusive motions has been investigated in a number of experimental and theoretical studies [5,8,15,31,32]. A parallel departure from the low-temperature harmonic trend, also known as dynamic transition, was found at about 200 K in both protein and protein hydration water mean square displacements [5,7]. The dynamic transition has been ascribed to the effects of the so-called second critical point of water [33]. The latter is observed by molecular dynamics simulations in the super-cooled phase of bulk water around 200 K, and marks the structural transition from a low-density liquid to a high-density one [34]. In this picture, protein hydration water undergoes a first-order transition at 220–230 K, where the low- to high-density liquid transition is related to a dynamic crossover from an Arrhenius to a super-Arrhenius temperature dependence of the relaxation times. However, this low- to high-density transition hypothesis to

* Corresponding author.
E-mail address: alessandro.paciaroni@fisica.unipg.it (A. Paciaroni).

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explain the dynamic crossover has been recently questioned by some experimental works [35–37].

All the studies mentioned above are devoted to investigate the diffusive motions of protein hydration water. On the other hand, little is known about the behaviour of vibrational excitations in the meV energy range — or the THz frequency range — when the temperature increases from the harmonic regime and crosses the protein dynamic transition. These excitations are usually described in terms of hydrogen-bond bending (HBB), hydrogen-bond stretching (HBS) and librational (LIB) modes, located at about 6, 22 and 80 meV, respectively [38,39]. Quite interestingly these vibrational features have been recently put in relationship with the hydrophobic/hydrophilic character of the biomolecule groups interacting with the water molecules [40].

Here we report a neutron scattering time-of-flight experiment performed on samples of perdeuterated maltose binding protein (MBP) hydrated in H_2O, so as to access directly the MBP hydration water signal. MBP is a well studied model protein that plays an important role in the metabolism of *Escherichia coli* [41], e.g. in the energy-dependent translocation of maltose and maltodextrins through the cytoplasmic membrane. Five spectral components have been singled out from the signal of protein hydration water: the elastic, quasielastic, HBB, HBS and LIB contributions. In order to thoroughly investigate their temperature dependence, these spectral features have been analysed by using the dynamic structure factor representation to focus on the low-energy range and the dynamic susceptibility to better emphasize the properties of the high-energy modes. We have found that the onset of anharmonicity connected with the dynamic transition, that is revealed in the elastic and quesielastic components, mostly affects the HBS band.

2. Materials and methods

2.1. Sample preparation

MBP is a two-domain protein, responsible for maltose uptake, that plays a major role in the signal transduction cascade that leads to chemotaxis. The two domains of the protein are of roughly the same size and are connected via a short helix and a two-stranded sheet [42]. Perdeuterated MBP was provided by the ILL-EMBL Deuteration Laboratory (D-Lab) in Grenoble. In the adopted protocol, MBP was produced as a histidine-tagged fusion protein, which allowed its purification by immobilized metal-ions affinity chromatography in a one-step procedure. The protein was expressed in high-cell density cultures and then purified. Thanks to such a procedure, a noticeable amount of MBP(D) was made available (about 200 mg). The purified MBP(D) sample was then left for 3 days in H_2O solution to exchange most of the labile deuterium atoms. In fact, as it turns out from NMR measurements, 601 hydrogen atoms out of a total number of 2961 in the MBP molecule are exchanged against deuterium [43]. The solution was dialyzed twice against the buffer, lyophilized and then dried under vacuum in the presence of P_2O_5 to remove the residual water. The MBP powder sample was finally hydrated by H_2O vapour pressure up to a hydration degree $h = 0.37$ g of water/g of dry protein.

2.2. Incoherent neutron scattering

Thermal neutrons are a powerful and widely-used probe to directly obtain information on the fast (nano- and pico-second) motions of biological samples. In neutron scattering experiments such motions are investigated by measuring the dynamic structure factor $S(\mathbf{Q},E)$, which represents the probability of an incident neutron to be scattered by the sample with an energy transfer E and a momentum transfer $\hbar\mathbf{Q}$ [44]. In the case of isotropic samples like the present one, the dynamic structure factor depends only on the wave vector modulus Q.

Hydrogen atoms have a very large incoherent neutron cross-section, that overcomes by far both its coherent component and the mainly coherent cross-sections of all other protein atoms, including deuterium. Thus, within the incoherent approximation, the signal of the MBP(D)-H_2O sample is dominated by the hydration water contribution, which indeed accounts for about 72% of the total scattering intensity, considering also the fraction of exchanged hydrogen atoms [43].

2.3. Neutron scattering experiment

Incoherent neutron scattering measurements were performed at the high-flux time-of-flight spectrometer IN5 at ILL (Grenoble, France). An energy resolution with half-width at half-maximum of $\delta E = 0.055$ meV at an incident wavelength $\lambda = 5$ Å was achieved in the elastic wave vector range $0.4 \le Q \le 2.2$ Å^{-1}. With time-of-flight spectrometers, one measures the number of neutrons $I(2\theta,t)$ that are scattered by the sample at an angle 2θ and arrive at the detector after a time t. As both the sample-to-detector distance and the neutron incident energy are known, $I(2\theta,t)$ can be easily transformed to obtain the angle-dependent dynamic structure factor $S(2\theta,E)$. By proper interpolation to take into account the kinematic constraints between Q, 2θ and E, $S(2\theta,E)$ can be then turned into the real dynamic structure factor $S(Q,E)$.

The sample was placed in a slab aluminium cell, at an angle of 135° with respect to the incident neutron beam, and measured in the temperature range $50 \le T \le 300$ K. A standard reduction procedure, taking into account empty cell contributions, sample transmission and angular dependence of the detector efficiency, was employed to treat the collected data. The value of the transmission coefficient was about 0.95. Multiple scattering was neglected.

3. Results and discussion

As recalled above, the dynamic structure factor $S(Q,E)$ of hydrated perdeuterated MBP, which contains information about the sample structure and dynamics, is dominated by the incoherent contribution of the hydration water protons; such a quantity, which is shown in Fig. 1a as a function of energy, at fixed wave vector $Q = 2$ Å^{-1} and for 6 different measured temperatures, allows us to carefully investigate the low-energy features of protein hydration water. Each spectrum may be considered as the sum of three different contributions, i.e. the elastic, quasielastic and inelastic signals. The different spectra components are listed in Table 1 for the convenience of the reader. The elastic peak, centred at zero exchanged energy, has an apparent finite linewidth defined by the experimental energy resolution δE, which corresponds to motions with characteristic time scales of about $\tau = \delta E^{-1} \approx 12$ ps. For Q values smaller than the inverse of the characteristic mean square displacements of water protons $\langle u^2\rangle_w$, the elastic intensity can be reasonably described through a Gaussian dependence $S(Q, E \approx 0) \approx \exp(-\langle u^2\rangle_w Q^2/3)$, by which one can directly estimate $\langle u^2\rangle_w$ [5]. In the low-temperature range this trend comes from the small-amplitude harmonic vibrational motions of water hydrogen atoms, therefore it corresponds to the so-called Debye–Waller factor of an ensemble of quantized harmonic oscillators [44]. From the Gaussian Q-dependence it can be inferred that the departure of the elastic intensities from unity gives a qualitative estimate of the water molecule mobility. This behaviour corresponds to the fact that when the temperature is increased, initially frozen motions with characteristic times faster than τ become active and appear outside of the energy resolution window, thus producing the elastic intensity decrease as the energy-integrated intensity of each spectrum is constant within the incoherent approximation [44]. In the inset of Fig. 1a we show the temperature dependence of the measured elastic intensity integrated over the investigated Q range. In the low-temperature range, the harmonic linear increase of $\langle u^2\rangle_w$ reflects on a smooth decrease of the elastic intensity. On the other hand, when temperature increases and roto-translational anharmonic diffusive motions of water molecules start taking place, $\langle u^2\rangle_w$ values become larger and larger, thus causing a more visible drop of the elastic intensity. As we mentioned above, such a decrease

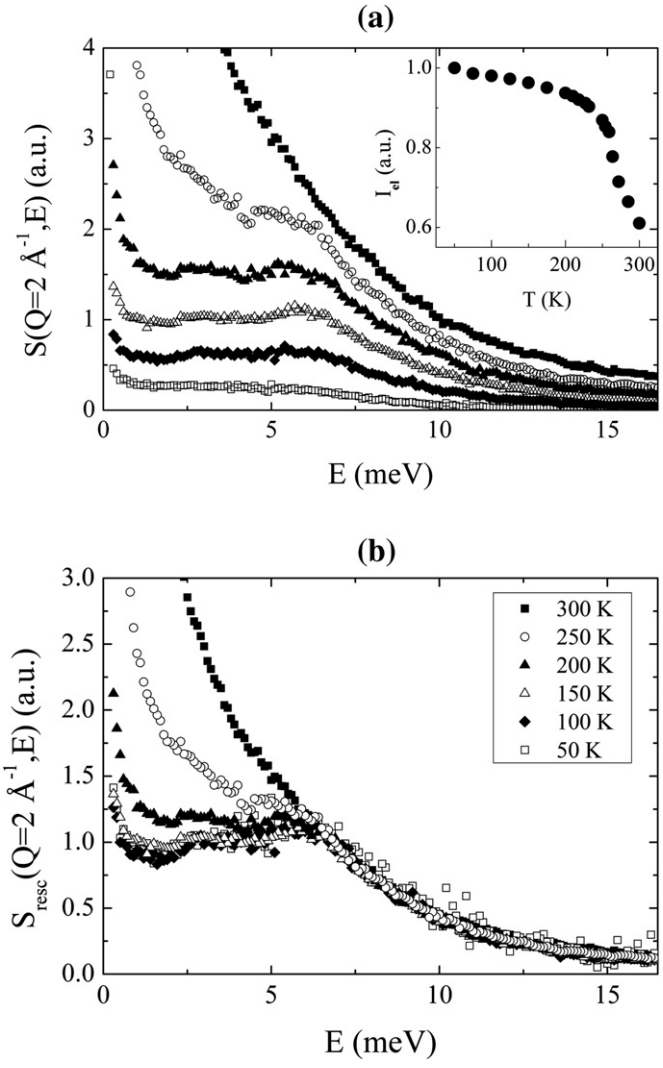

Fig. 1. a) Dynamic structure factor of MBP hydration water at selected temperatures and at constant wave vector Q = 2 Å^{-1}. Temperature values are shown in the legend of the panel b). Inset: temperature behaviour of the elastic intensity, as obtained by integrating the elastic peak over the energy resolution width (FWHM). The integrated elastic intensities have been normalized by setting a unitary value for the lowest temperature point. b) Dynamic structure factor rescaled by Debye–Waller and Bose factors, from the original temperatures of each measurement to the common temperature of 50 K.

is compensated for by a corresponding rise of quasielastic and inelastic scattering. The quasielastic signal, whose tails extend up to about 5 meV [13], appears as a further broadening of the elastic peak, due to diffusive modes. The inelastic signal is instead brought about by the density of states (DOS) of the sample's vibrational modes.

Table 1
List of the components in protein hydration water spectra with the corresponding assignments and energies.

Spectral components	Energy (meV)	Energy (cm^{-1})
Elastic [13]	−0.055 ÷ 0.055	−0.44 ÷ 0.44
Quasielastic (QES) [13]	0.055 ÷ 5	0.44 ÷ 40
Protein boson peak (BP) [53]	~3	24
Hydrogen-bond bending (HBB) [38]	6–7	48–56
Hydrogen-bond stretching (HBS) [38,39]	~22	~176
Librations (LIB) [38,39]	80	640

In the Debye approximation, the E^2-dependence of the DOS low-energy region is removed from the incoherent dynamic structure factor $S(Q,E)$ by two other factors appearing in the inelastic one-phonon harmonic scattering cross section, namely the Bose occupation number $n(E,T) \sim k_B T/E$ and the $1/E$ factor [44]. A Debye-like DOS should therefore contribute as a constant to $S(Q,E)$. Instead, the spectra of hydration water shown in Fig. 1a display, in the low-frequency region, the signature of at least two extra intensities with respect to the simple Debye-like behaviour, namely at about 3 and 6 meV. These two structures are well-visible below 250 K. As the temperature increases they become less and less distinguishable, owing to the rise of the quasielastic contribution which gradually overwhelms the inelastic signal.

The first signal around 3 meV is present as a well-prominent bump also in the incoherent neutron scattering spectra of both wet and dry hydrogenated proteins, although slightly shifted to lower energies in the second case. For instance, it has been reported for a number of proteins such as lysozyme and myoglobin [45], azurin [46,47], β-lactoglobulin [12] and green fluorescent protein [7], with this list being far from exhaustive. In all the above cases, such a low-energy peak in proteins has been correlated to the anomalous excess of DOS modes known as Boson peak (BP) in the domain of glassy systems. Despite our spectra being dominated by the incoherent scattering of the hydration water protons, the smaller coherent signal arising from the deuterium atoms of the protein can still produce a visible, albeit small, contribution to the dynamic structure factor. In addition, such a signal may come from the fraction of protein deuterium atoms exchanged against hydrogens, that turns out to be about 20% of the total number of MBP deuterium atoms [43]. Another mechanism — correlated to the previous one — that can further enhance the intensity of the 3 meV bump arises from a protein–water dynamic coupling in the involved low-energy range. In fact, MD simulations predict the existence of a BP in the spectrum of hydration water itself, with an amplitude that tends to disappear when the α-C atoms of the protein backbone are progressively fixed [15].

The second bump around 6 meV can instead be ascribed to the HBB mode of hydration water. Indeed, a peak at 6 meV is also a peculiar feature of the vibrational DOS of bulk liquid [48–50] and supercooled water [51]. In addition, it is reminiscent of the transverse acoustic mode TA1 sustained by the O–O–O bending mode in crystalline ice [52]. More recently, neutron scattering measurements have shown that the same feature appears also in the DOS of protein hydration water in the "solid" phase (at 100 K), where it displays a lineshape similar to that of amorphous ice [53]. Such a comparison between the vibrational dynamics of bulk and amorphous water has been more deeply investigated by a MD simulation work. The authors show that bulk water supercooled down to 200 K also possesses a BP at about 4.6 meV, which progressively shifts towards higher energies (~6 meV) when the system is further cooled down to the low-density amorphous ice region [50]. On the other hand, a similar mode has been found also in the dispersion curves of bulk water measured by inelastic neutron [54] and X-ray scattering [55]. The close relationship between the BP of a glassy system and the transverse acoustic van Hove singularity in the crystalline counterpart, i.e. the 6 meV peak of crystalline ice in the present case, has been recently pointed out by Chumakov and co-workers [56].

In summary, while the 3 meV bump is likely connected to the BP of the protein, the second bump at 6 meV can be ascribed to the HBB mode belonging to the true vibrational DOS of (hydration and bulk) water, probably already beyond the domain of applicability of the Debye approximation.

Further insights about the nature of the two observed bumps can come from a closer inspection of the temperature evolution of the spectra. Particularly, the presence of anharmonic signatures in the low-energy spectral range can be highlighted by rescaling the curves by the terms that in the harmonic one-phonon approximation depend on the temperature, i.e. the Bose occupation factor $n(E,T)$ and the Debye–Waller factor [44]. This has been done by rescaling all the data in

Fig. 1a to the lowest measured temperature $T_0 = 50$ K by the relationship [46]:

$$S_{resc}(Q,E) = S(Q,E)\frac{n(E,T_0)\exp\left(-<u^2(T_0)>_w Q^2/3\right)}{n(E,T)\exp\left(-<u^2(T)>_w Q^2/3\right)} \quad (1)$$

where the Debye–Waller factors have been calculated by fitting the temperature dependence of the elastic intensity with the expression

$$I_{el}(T) = I_{el}(T_0)\exp\left(-<u^2(T)>_w Q_{av}^2/3\right) \quad (2)$$

where $<u^2(T)>_w = \frac{E}{2K}\coth\frac{E}{2K_BT} - <u^2(T_0)>_w$ corresponds to the mean square displacements of an ensemble of quantized harmonic oscillators as in an Einstein solid [44] and $Q_{av} = 1.3$ Å^{-1} is the average wave vector [44]. The resulting spectra are plotted in Fig. 1b. Between 50 and 150 K the data rescale quite well in all the explored energy range. Above 150 K the onset of quasielastic scattering, due to the temperature activation of diffusive motions, coincides with the crossover to the anharmonic regime of the MSDs discussed in the introduction and related to the so-called dynamic transition of hydration water. On the other hand, for energies above 6 meV, the spectra remain well superimposed up to room temperature, thus indicating that in the dynamic structure factor representation the vibrational features of hydration water seem to have a rather harmonic behaviour beyond the region of the protein BP. Actually, we will see below that small departures from harmonicity are observable also at energies higher than the BP. The fact that the 6 meV peak would mark the threshold between a low-energy anharmonic glass-like behaviour and a high-energy harmonic crystal-like behaviour is consistent with the above discussion about the nature of the two observed bumps. This is also supported by MD simulation results showing that the BP of supercooled water is blue-shifted, and finally localizes at the crystal-like energy of 6 meV, when the liquid structure is made progressively harmonic [57].

Possible deviations from a harmonic temperature evolution, even up to frequencies higher than the involved thermal energies, can be better highlighted by representing the spectra in terms of the dynamic susceptibility, whose imaginary part χ'' is related to the dynamic structure factor by the fluctuation–dissipation theorem [44]: $\chi''(2\theta,E) = \pi\, S(2\theta,E)/[n(E)+1]$. From this definition it is clear that the dynamic susceptibility still contains a residual temperature dependence from the Debye–Waller factor. We also remark that $\chi''(2\theta,E)$ is calculated from the scattering angle-dependent function $S(2\theta,E)$ and not from $S(Q,E)$, because in this latter case the limited accessible dynamic range would not allow to investigate the high-energy range at a fixed Q value. The temperature dependence of the dynamic susceptibility, calculated from $S(2\theta,E)$ averaged over all the angular range and corresponding to a mean scattering angle of 65° (equivalent elastic Q = 1.3 Å^{-1}), is plotted in Fig. 2. At a first inspection, the experimental susceptibility appears immediately composed of various spectral bands that display rather different temperature behaviours. The first band lies within the elastic resolution peak, between 0 and ~0.2 meV, and reproduces the decreasing trend already shown in the inset of Fig. 1a discussed above. Other four major bands can be singled out in the susceptibility spectra, namely between 0.2 and few meV, around 7 meV, between roughly 15 and 40 meV, and around 80 meV.

The low-energy band between 0.2 and few meV arises from quasielastic scattering and displays the strongest temperature dependence. In the liquid phase at high temperature, this band is mainly due to diffusive motions of the water molecules, of both translational and rotational nature. At temperatures below 0 °C, the hydration water mobility is rapidly reduced, although it is not abruptly quenched as one would expect upon passing through a liquid-to-solid phase transition. It is indeed well known that, due to the interaction with the protein surface, hydration water is in a confined-like state that presents several dynamic and structural similarities with the supercooled liquid or the glassy solid

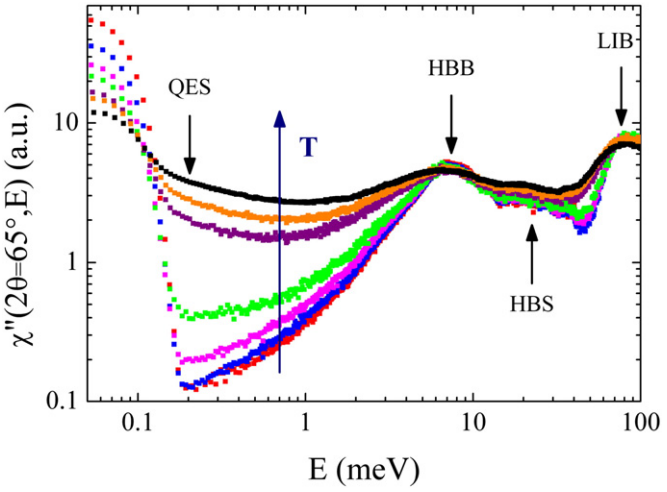

Fig. 2. Dynamic susceptibility of MBP hydration water at constant scattering angle 2θ = 65° and different temperatures, namely 100 (red squares), 150 (blue squares), 200 (pink squares), 250 (green squares), 264 (purple squares), 272 (orange squares) and 300 K (black squares). At each temperature, a cut-off of about $5k_BT$ was applied. For the sake of clarity each component has been labelled as follows: quasielastic scattering (QES), hydrogen-bond bending (HBB), hydrogen-bond stretching (HBS) and librational (LIB) modes.

[57]. In the past, such a low-energy band was detected by a number of spectroscopic techniques also in bulk water: in the supercooled region down to 260 K, the analysis of its temperature dependence shows that the band can be ascribed to a slow structural relaxation, well-known in glasses as α-relaxation process in the framework of the mode coupling theory [51]. Such a picture of bulk supercooled water can be applied to interpret our neutron susceptibility data of hydration water. Upon cooling below the nominal freezing point, the diffusive dynamics is suppressed but, due to the confining effects of the protein, hydration water enters a supercooled regime where glass-like diffusive motions still take place and are then progressively slowed down with further decreasing the temperature. Actually, this phenomenon can be very general and was observed also when localized and confined translational diffusive motions are decoupled from the α-relaxation process, such as in the case of β-relaxation [37].

The bands at higher energies are known to arise instead from motions of vibrational nature. In fact, hydrogen-bond network excitations located around 7, 22 and 80 meV are usually assigned to the HBB, HBS and LIB modes, respectively [38,39]. Note that in the susceptibility representation the HBB band is slightly blue-shifted with respect to the position found at 6 meV in the $S(Q,E)$, due to the factor $1/[n(E)+1]$ contained in the definition of $\chi''(Q,E)$. We also remark that the position of 22 meV for the HBS band is only approximative, as in our neutron scattering data this component appears as a quite broad bump. The role played by the HBB, HBS and LIB vibrational bands with respect to the protein dynamic transition is indeed the main scope of this paper.

In Fig. 2, the HBB band at 7 meV displays no significant temperature dependence, apart from a weak broadening and a slight decrease of intensity with increasing T. The same applies to the LIB band around 80 meV, which undergoes a small intensity variation only between 300 and 250 K, whereas no further evolution is visible at lower temperatures. The broad band between 15 and 40 meV, which is connected with the HBS modes around 22 meV, seems to be more affected by temperature than the other bands.

For a more quantitative insight onto the temperature behaviour of the above four bands, the susceptibility was integrated over an energy window ΔE around the nominal energy positions $\bar{E}$ of each band. For $\bar{E}$ equal to 0.25, 7, 22 and 80 meV, the integration window ΔE was fixed at 0.1, 1, 4 and 8 meV respectively. The resulting intensities are plotted as a function of temperature in Fig. 3. As it can be noticed in panel a), the

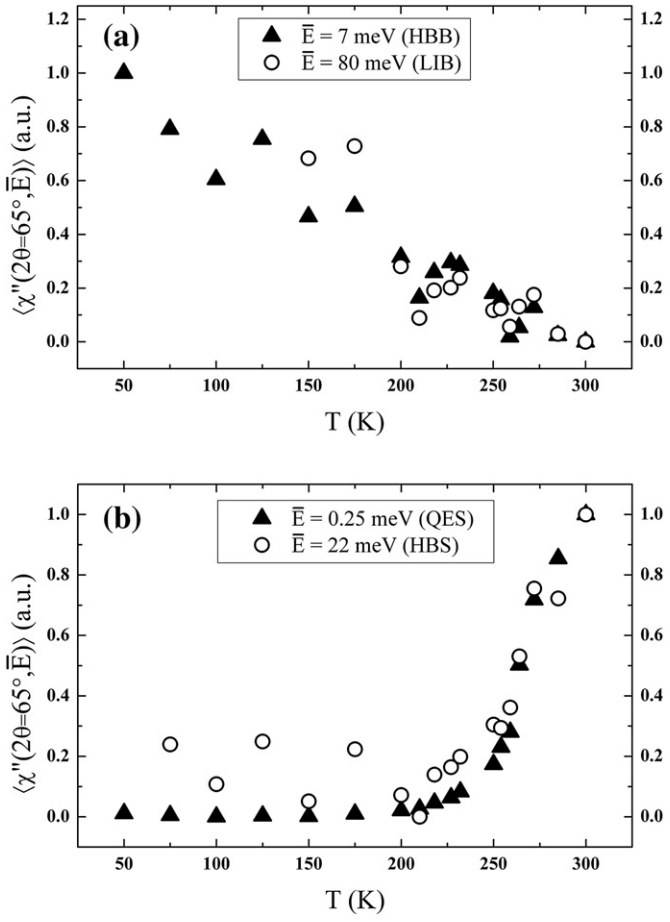

Fig. 3. Temperature dependence of the susceptibility integrated intensities for (a) HBB and LIB modes, at 7 and 80 meV respectively, and (b) diffusive and HBS excitations, located at 0.25 and 22 meV respectively. The integrated intensities have been normalized between 0 (minimum value) and 1 (maximum value).

trend of the HBB and LIB bands confirms that these modes are very weakly affected by temperature variations and display a linear decreasing behaviour. As we mentioned above, in the one-phonon harmonic approximation, the only dependence on the temperature in the susceptibility function comes from the Debye–Waller factor [44] which results in the slightly negative slope observed in Fig. 3a. In all cases, the most apparent and interesting result is the absence of any kind of break around the dynamic transition temperatures, i.e. between 200 and 250 K.

The temperature behaviour of the diffusive and HBS bands is very different, plotted in Fig. 3b. As anticipated above in general terms, the quasielastic scattering region, where the diffusive band lies, provides the major contribution to compensate for the elastic intensity drop at high temperatures. More specifically, between 200 and 250 K the temperature trend of the diffusive process presents a marked deviation from the flat low-temperature level. A comparison with the inset of Fig. 1a reveals that the elastic intensity loss, that is connected with an enhancement of the molecular mean-squared displacements (MSDs), deviates from the linear low-temperature behaviour in exactly the same temperature range. From a closer inspection it is clear that elastic intensity drop and quasielastic intensity gain follow and complement each other rather faithfully. Wood and co-workers revealed, by elastic incoherent neutron scattering, that protein hydration water undergoes a dynamic transition at the same temperature where the dynamic transition of its protein takes place [5], thus highlighting the existence of a strong dynamic coupling between the biomolecule and the surrounding solvent. This finding is now complemented by the present observation that — in hydration water — the enhanced MSD above the transition are generated to a significant extent by the activation of diffusive motions, whose characteristic timescale was shown to be about 14 ps at room temperature [13].

Similar considerations apply to the last band under analysis, i.e. the HBS modes distributed around 22 meV. Indeed, the corresponding integrated susceptibility, in Fig. 3b, also shows a thermally activated behaviour, with a low-temperature flat trend followed by a steep increase at higher temperatures. Although the relative variation is globally smaller than for the diffusive band, the dynamic onset occurs again in the temperature range between 200 and 250 K. When properly rescaled, the intensities of the HBS modes and the diffusive process superimpose rather well in the whole temperature range. Quite interestingly, from a closer look into the temperature trend of the elastic, quasielastic and HBS intensities, we can recognize a two-step regime with a first break at 200 K and a steeper increase at about 250 K. These two critical temperatures can be ascribed respectively to the glass transition of the hydrated protein system and to the solvent dynamic transition, occurring when the relevant diffusive process enters into the timescale of the spectrometer [58]. For example, in the case of MBP and its hydration water measured on different spectrometers [59], a derivative analysis of the elastic intensity trend pointed out a first onset always located at the calorimetric T_g (slightly below 200 K), and a second, sharper one, occurring at different temperatures depending on the energy resolution of the spectrometer (for IN5 it is around 250 K).

As to the first departure from the low-temperature trend of the quasielastic intensity, it is noteworthy that the same phenomenon has been observed by Raman spectroscopy on crossing T_g in proteins and related solvents, and was closely ascribed to changes in the Boson peak frequency and intensity [60]. Actually, a neutron scattering study of hydrated DNA by Sokolov and co-workers [61] reported for the low-energy band integrated over a similar energy range as above, a change of intensity at around 180–200 K. From the data transformed into frequency dependent susceptibility spectra $\chi''(\nu)$, it is clear that no α-peak is active over that dynamic range at those temperatures. On the contrary, $\chi''(\nu)$ is rather dominated by a nearly constant (power law) loss (NCL), whose intensity shows a crossover around 180–200 K. This phenomenon parallels what happens to NCL in many glass formers [62] and is a general property of caged dynamics.

Concerning instead the so-called dynamic transition, which occurs at higher temperature and depends on the spectrometer resolution, it originates from hydration water localized motions that share the character of the water Johari–Goldstein β-relaxation entering the time window of the spectrometer, and in some cases can be decoupled from the timescale of the cooperative α-relaxation [37,59]. The observed anharmonic onset of the HBS vibrational dynamics would be thus related to the appearance of such a β-relaxation contribution, in analogy to what happens in polymer systems, where a close relationship between low-frequency vibrational dynamics from local processes and the β-relaxation was observed also by Fourier transform infrared spectroscopy [63–66].

The globally emerging picture indicates that, among the four investigated spectral regions of the hydration water susceptibility, the LIB and the HBB band are very slightly affected by temperature upon crossing the protein–water dynamic transition. These two bands can be considered to maintain a (quasi-)harmonic character in all the explored temperature intervals. Indeed the only signature of a small anharmonic behaviour appears as a slight broadening of their energy widths at higher temperatures. On the contrary, the diffusive and HBS bands display a strongly anharmonic temperature evolution, as they become remarkably enhanced at and above the dynamic transition temperature. This finding suggests that these types of motions are significantly involved in the transition itself. In this respect, it is particularly interesting to remark that HBS modes, given the nature of the corresponding polarization vectors, are considered to be responsible for the propagation of longitudinal acoustic-like collective modes, in both bulk [54] and hydration water [67]. By comparing quasielastic incoherent neutron scattering

with MD simulations, Wood and co-workers [5] suggested that the MSDs responsible for the dynamic transition in hydration water are due to diffusive processes of mainly translational nature, while rotational diffusion would play a minor role, in agreement with previous numerical results [31]. In addition to this, our inelastic incoherent neutron scattering results directly show that (*i*) the dynamic transition in hydration water is contributed also by purely vibrational modes and, among such modes, (*ii*) the major role is played by the longitudinal ones, which are by definition of translational nature as well. We can then affirm that, in the protein hydration shell, the enhanced MSDs are due to an increased molecular mobility mainly produced by the activation of translational diffusive motions, with an additional contribution from the onset of anharmonic hydrogen-bond stretching oscillations. Such anharmonic HBS modes allow the water molecules to sample a larger region of their potential energy well and to move further away from their equilibrium position. We can speculate that the latter might provide the first necessary steps for the water molecules to start their diffusive motions and jump towards other neighbouring potential wells. In this picture, the onset of sub-picosecond HBS modes would trigger diffusive motions, whose translational character is indeed consistent with the longitudinal nature of hydrogen-bond stretching vibrations. On the contrary it is reasonable to suppose that rotational diffusion would rather imply the activation of hydrogen-bond bending oscillations.

Our observations confirm what was guessed by Doster and co-workers [68,69], who compared infrared spectra of the O–H(D) stretching vibration and volumetric data of protein-adsorbed water as a function of the temperature. They found a discontinuous change in the thermal expansion coefficient at the calorimetric glass temperature T_g, accompanied by a kink in the intensity of the stretching band vs. temperature, pointing out a strict relation with what happens around 200 K to the elastic scattering intensity of H_2O-hydrated deuterated phycocyanin. In that context, they hypothesized that "a critical number of open bonds controlled by the β-process is a prerequisite for the α-relaxation to occur". In other words, the onset of sub-picosecond HBS modes, controlling the opening of H-bonds, can allow motions over the neighbour cages. In our case, the phenomena are mainly reflecting water dynamics and the broad dynamic range investigated with the same technique allows to highlight for the first time the connection between HBS modes and diffusive motions.

4. Conclusions

The present neutron scattering results give new insights into the temperature dependence of the inelastic features of protein hydration water. The dynamic structure factor shows two large peaks at about 3 meV and 6 meV, which can be probably ascribed to a residual contribution of the protein dynamics and to the HBB mode of water, respectively. The intensity of both peaks rescales quite well with the Bose–Einstein factor at low temperatures. More interestingly, the trend of the dynamic susceptibility reveals that the HBB and the LIB modes show no sign of break in correspondence of the dynamic transition of protein hydration water, that is instead highlighted by the behaviour of the elastic peak intensity. The opposite is true for the HBS mode, that parallels the temperature dependence of the quasielastic intensity and is correlated with the water dynamic transition. Actually, even if this transition manifests itself mostly with the abrupt decrease of the elastic intensity and the rise of the quasielastic signal, in the present case we find evidence that also other dynamic degrees of freedom, such as the HBS collective bands at 22 meV, are involved. We suggest that HBS motions also contribute, although to a lesser extent, to the onset of water MSD above the transition. Such anharmonic longitudinal HBS modes would correspond to the early dynamic stages during which water molecules can sample larger distances from their vibrational equilibrium positions, before starting their translational diffusive motions by jumping to neighbouring sites. The enhanced HBS motions can have a role for biological functionality, as they can be coupled to fast vibrations of protein surface groups that are key to a number of processes, such as recognition and binding of ligands. The nature and the extent of this vibrational coupling is a subject of major interest that deserves to be investigated in the future.

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