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Low-frequency vibrational modes in proteins: a neutron scattering investigation

Abstract The low-frequency dynamics of plastocyanin, an electron transfer copper protein, has been investigated by incoherent neutron scattering at different temperatures. The contribution to the dynamic structure factor arising from H/D exchangeable and non-exchangeable protein protons has been evaluated by analyzing two differently exchanged protein samples. The dynamic structure factor of a hydrated plastocyanin sample with all the exchangeable hydrogens (about 150) replaced by deuterium exhibits an excess of vibrational modes, at about 3.5 meV, reminiscent of the boson peak found in other proteins and glassy systems. When only fast exchangeable hydrogens (about 50) are substituted by deuterium, the protein, besides the above-mentioned peak, shows an additional peak at about 1 meV. These vibrational peaks are discussed in connection with the topological disorder of the systems and the fluctuations of the intramolecular hydrogen bonds.

Keywords Protein dynamics · Boson peak · Plastocyanin

Introduction

Proteins display a very complex structure which is reflected in a huge variety of motions crucial in determining their biological activity. These motions cover a wide temporal window (from femtoseconds to even seconds), extending from fast vibrations of atoms or groups of atoms, to slow collective motions involving a large portion of the protein macromolecule. It is com-

monly believed that a minimum amount of water is required to activate the dynamics of the protein, allowing it to reach full biological functionality (Rupley et al. 1983).

Different experimental investigations have provided evidence that a hydrated protein behaves as a harmonic solid below a critical temperature. Above it, the onset of anharmonic motions gives rise to a dynamic transition; such a glass-like dynamic behaviour has been established by investigations of many conformational substates and local minima of the potential energy (Frauenfelder et al. 1988; Iben et al. 1989).

The dynamic structure factor, as measured by inelastic neutron scattering and Raman spectroscopy, reveals the presence, in most of the protein systems investigated, of a low-frequency vibrational peak, centered at about 2–3.5 meV (Diehl et al. 1997 and refs. therein; Paciaroni et al. 1999a). Such an excess of modes, with respect to the flat Debye level, represents a typical anomaly found in glassy systems (Frick and Richter 1995), probably connected to their low-temperature anomalies (Phillips 1981), and has been named the boson peak. The origin of such a spectral feature, and its possible implication in biological functionality, is up to now widely debated. The evidence that water depresses the inelastic neutron scattering intensity of such a peak in proteins led some authors to hypothesize the involvement of the solvent-exposed lateral chain librations (Diehl et al. 1997). Moreover, the presence of a similar feature in partially denatured myoglobin, as well in a mixture of hydrated amino acids (Diehl et al. 1997), has suggested that the corresponding vibrations are independent of the main-chain conformation and packing and of the monomer chemical linkage (Cusack and Doster 1990). More generally, it has been hypothesized that, in analogy with glasses, the boson peak in proteins might originate from the topological disorder of the system, which could give rise to scattering of acoustic phonons (Paciaroni et al. 1999b).

The nature of the boson peak has been faced also by different theoretical and computational approaches.

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Normal mode analysis performed on some protein macromolecules showed the presence of a number of delocalized vibrational modes with frequency < 2 meV; such modes involve collective macromolecular displacements of possible biological relevance (Seno and Go 1990; Diehl et al. 1997). The modes corresponding to the vibrational peak have been also interpreted in terms of accordion-like modes of the α-helices and of the β-sheets (Chou 1985), and their frequencies have been calculated for several proteins in the range 2.5–3.5 meV. Moreover, Go (1978) interpreted the low-frequency spectrum of a protein as the superposition of the vibrational contributions of an elastic sphere, whose characteristic frequencies should be inversely proportional to the radius of the sphere. However, such a dependence has not been experimentally observed (Painter et al. 1982).

Molecular dynamics (MD) simulations revealed the presence in myoglobin (Steinbach et al. 1991) and azurin (Paciaroni et al. 1999a) of a low-frequency peak centered at about 1.5 meV. The origin of the discrepancies in the energy position of the boson peak between the simulated and the experimental spectra has been tentatively attributed to an inaccurate force field related to the electrostatic cutoff or to the intermolecular interactions (Smith 1991; Steinbach et al. 1991; Melchionna and Desideri 1999). Even if an improvement of the description of the experimental dynamic properties of the system is of utmost relevance, the MD simulation approach offers the possibility of investigating, with atomic resolution, the origin of the boson peak through a reliable assessment of the contribution of each protein hydrogen to the incoherent inelastic neutron scattering. Indeed, a MD simulation screening of the protein protons has allowed us to propose a significant contribution to the boson peak from the protein backbone hydrogens (Paciaroni et al. 2000).

Therefore, an experimental assessment of the protein structural elements whose dynamic contribution is significant to the boson peak is highly desirable, also in connection with a similar vibrational anomaly recently observed, by neutron scattering, in the hydration water surrounding a protein macromolecule (Paciaroni et al. 1999b); such a feature had been predicted by MD simulations performed on the same system (Paciaroni et al. 1998). Indeed, a possible intriguing dynamic coupling between the protein and the surrounding solvent was suggested by these authors.

We have carefully analyzed, by neutron scattering, the incoherent dynamic structure factor of plastocyanin (PC), a copper-containing electron transfer protein involved in the photosynthetic process, submitted to appropriate hydration procedures to substitute, by deuterium, the exchangeable protein protons. The analysis of the dynamic structure factor shows that the sample in which all the exchangeable hydrogens have been replaced by deuterium reveals a vibrational peak, centered at 3.5 meV. Together with such a peak, an additional narrower peak, centered at about 1 meV, is

observed in the sample in which only the fast exchanging hydrogens have been substituted. These results may give new insight into the low-energy vibrational dynamics of protein systems.

Materials and methods

An incoherent neutron scattering experiment reveals the variation of the scattered intensity as a function of the momentum ($\hbar q$) and energy transfer ($h\nu$), the measured quantity being the dynamic structure factor $S(q,\nu)$. If we take into account only the contribution of hydrogen atoms, in the one-phonon scattering approximation, $S(q,\nu)$ can be separated into an elastic, a quasielastic and an inelastic part:

$$S(q,\nu) = DWF(q,T)\big[EISF(q)\delta(\nu) + (1 - EISF(q))S_{qel}(q,\nu)\big]$$
$$+ DWF(q,T)\frac{q^2}{8\pi M\nu}n(\nu,T)g(\nu) \qquad (1)$$

where $DWF(q,T)$ is the Debye-Waller factor, $EISF(q)$ is the elastic incoherent structure factor, $S_{qel}(q,\nu)$ represents the broadening of the elastic peak, M is the proton mass, $n(\nu,T)$ is the Bose factor and $g(\nu)$ is the vibrational density of states. The first term in Eq. 1 provides the elastic scattering, which gives information on the geometry of the volume accessible to the sample atoms at infinite time, while the second term corresponds to the quasielastic component and indicates the presence of non-vibrational, diffusive motions in the sample. The last term describes the inelastic contribution, resulting from exchange of energy with vibrational modes in the molecule.

The experiment was performed on the cold neutron multichopper time-of-flight spectrometer IN5 at the Institut Laue Langevin (ILL) using neutrons of 6 Å wavelength, resulting in an elastic resolution of about 60 µeV (full width at half height at the elastic peak), a range of q from 0.4 to 1.8 Å^{-1} and an accessible energy range from approximately –1.5 meV up to 50 meV (from energy loss to energy gain domain). In a time-of-flight spectrometer the time necessary to the scattered neutron to hit the detector bank is used to measure the exchanged energy during the scattering event, while from the angle of detection with respect to the incident beam 2θ the exchanged momentum can be derived. By transforming in energy the time-of-flight corresponding to the measured intensity, one can calculate the dynamic structure factor $S(2\theta,\nu)$. Moreover, we have derived the dynamic structure factor $S(q,\nu)$ by transforming $S(2\theta,\nu)$ into a constant wavevector q format, using an interpolation procedure to take into account the variation of q with ν at a fixed scattering angle because of the neutron kinematics.

The data were corrected using standard ILL programs to take into account incident flux, cell scattering, self-shielding and detector response, referring to a vanadium standard. To improve the counting statistics, the measured 81 spectra were binned into five groups covering a scattering angle range from 20° to 120°. Unfortunately, the detectors corresponding to $90° < 2\theta < 120°$ have shown spurious signals, probably due to electronic noise, and were discarded. Since an average transmission of 93% was obtained, multiple scattering or multiphonon corrections were not applied. The coherent contribution to the total signal has been evaluated to be about 15–20% for both the A and B samples (A. Paciaroni, unpublished results).

Spectra were accumulated for 10–12 h at the temperatures $T = 100, 220$ and 300 K. In all cases, an amount of about 400 mg of sample (protein plus hydration water) was held in a standard flat aluminium cell with internal spacing 0.5 mm, placed at an angle of 135° to the incident beam.

Poplar PC (molecular weight 10,500 Da) was obtained by chromatographic purification from *E. coli* cells where the PC gene had been cloned, according to reported procedures (Paciaroni et al. 1999a). The main contribution to the dynamic structure factor of proteins arises from the dominant incoherent scattering

of hydrogen atoms because of their large cross-section with respect to other nuclei. Since hydrogens are uniformly distributed within the whole macromolecule, they probe most of the diffusive and vibrational motions of proteins (Bèe 1988). In addition, owing to the much smaller neutron scattering cross-section of the deuterium atom (D) with respect to hydrogen (H), the contribution of the solvent to the spectrum, in a D_2O-hydrated protein, can be neglected. Each PC macromolecule contains 558 non-exchangeable protons, 48 solvent-exposed fast exchanging protons and 100 buried protons with a long exchanging time (Paciaroni et al. 1999a). Therefore, a controlled isotopic H/D exchange, conceived to remove (or not) slow exchanging protein protons, could allow us to single out the dynamic contribution to the neutron scattering from exchangeable and non-exchangeable protein protons. Accordingly, we prepared two D_2O-hydrated protein samples by following different procedures. First, an amount of about 350 mg of lyophilized PC was hydrated with D_2O at $h=0.40$ g of D_2O/g of protein in a chamber under vacuum in the presence of a saturated KCl deuterium solution. In such a way, all the rapidly exchanging hydrogens were substituted by deuterium atoms. We denote this as sample A.

To prepare sample B, lyophilized PC was dissolved in bulk D_2O at a concentration of about 0.5 mM and kept at 4 °C for 2 weeks. It was then lyophilized, dissolved again in D_2O and left in solution for 1 week by repeating such a procedure three times to substitute all the protein exchangeable protons by deuterium, even those with a slow exchanging time (the total number of exchanged hydrogens is 148). Finally, an amount of 350 mg of the lyophilized sample was dried in the presence of P_2O_5 under vacuum and then hydrated up to $h=0.40$ g of D_2O/g of protein by a saturated KCl deuterium solution. The residual amount of water, evaluated by IR spectroscopy, is practically the same in both the samples (A and B) and less than 0.05 g of water per g of protein, in agreement with that observed by Diehl et al. (1997).

Fig. 1 Dynamic structure factor, $S(2\theta,v)$, as a function of energy at a fixed scattering angle $2\theta=75°$, of D_2O-hydrated plastocyanin (PC) containing exchangeable and non-exchangeable protein protons (sample A: *open symbols*) and of D_2O-hydrated PC containing non-exchangeable protein protons (sample B: *filled symbols*) at 100 K (*squares*), 220 K (*triangles*) and 300 K (*circles*). The selected spectra were normalized to represent the intensity of a single protein macromolecule. *Inset*: the ratio between the signal of sample A and that of sample B at 100 K (*circles*), 220 K (*triangles*) and 300 K (*squares*) after a normalization to take into account the different number of scatterers in each sample
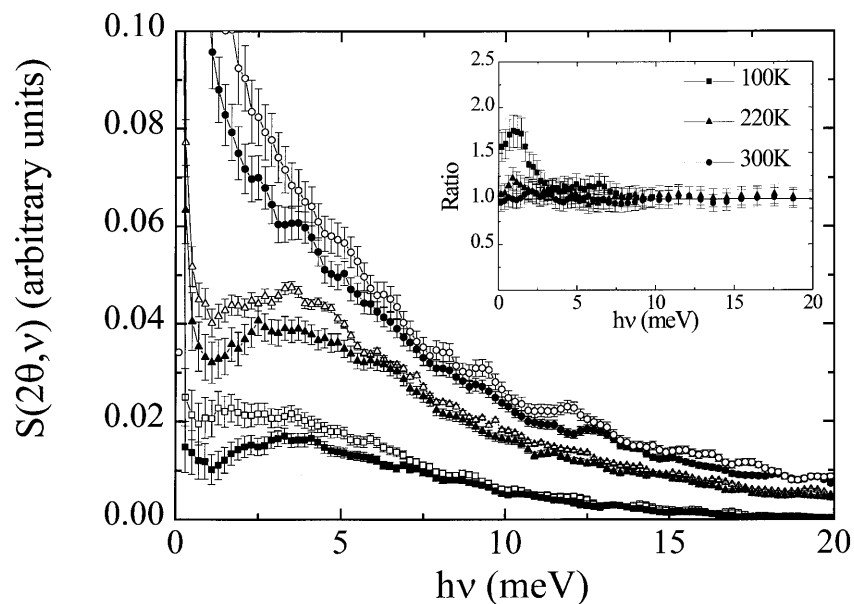
Results and discussion

The dynamic structure factor, $S(2\theta,v)$, of samples A and B was measured at three different temperatures. The results, at a fixed scattering angle of $2\theta=75°$, are shown in Fig. 1. The curves have been carefully normalized to the number of protein macromolecules present in each sample. We can immediately observe that the signal corresponding to sample A (empty symbols) is generally more intense than that of sample B (filled symbols), according to the larger number of hydrogens present in the former sample. Each spectrum can be considered as the sum of three different terms, i.e. the elastic, quasielastic and inelastic contributions (see Eq. 1), which usually show different behaviour as a function of temperature (Bèe 1988). We observed that the elastic intensity decreases upon increasing the temperature, according to the Debye-Waller factor behaviour, and such a decrease is compensated for by the quasielastic and inelastic scattering [these results are not shown, but are qualitatively the same as those reported for a similar protein, azurin (Paciaroni et al. 1999a)].

The quasielastic part results in the broadening of the elastic peak and indicates the presence of non-vibrational, diffusive motions in the sample and it is mostly visible at 300 K. We are mainly interested in the inelastic part of the spectrum, which results from exchange of energy with the molecule vibrational modes. In this region, we note a broad peak, centered at about 3.5 meV, in the dynamic structure factor measured at 100 and 220 K. Such a peak is present in both A and B samples, appearing more symmetric in sample B where, in addition, it is slightly less intense. At room temperature, such a feature almost disappears, possibly because of the rise of the quasielastic contribution. In addition, we note that sample B shows a well-defined minimum between the elastic peak and this boson peak

at 100 and 220 K. Conversely, extra intensity, which completely fills such a minimum at 100 K, but only partially at 220 K, appears in the spectrum of sample A; a similar feature has also been observed in dry myoglobin and dry lysozyme (Diehl et al. 1997). This is better emphasized in the inset of Fig. 1, where the ratio between the signal intensity of the A and B samples has been plotted as a function of energy, at the three temperatures, after an appropriate normalization to take into account the different number of scatterers in the two samples. At 100 K, besides slight oscillations at higher energy, this ratio shows a very distinct peak at about 1 meV. Such a peak becomes less intense at 220 K and disappears at room temperature, where the ratio levels to one, as occurs at all the temperatures for higher energies.

Additional information about the spatial dependence of the peaks comes from Fig. 2 in which the dynamic structure factor $S(2\theta,v)$ of both the A and B samples, at 100 K, is shown for three different 2θ values. First, we note that the differences between the $S(2\theta,v)$ of the A and B samples become progressively more evident by increasing the 2θ values. Actually, while at $2\theta = 40°$ the A and B spectra practically coincide within the error bars, but at $2\theta = 90°$ they are markedly different in the 0-8 meV range. In addition, we note that the 3.5 meV peak becomes more pronounced as far higher 2θ values are taken into account. It should be remarked that the observed inelastic peak at 3.5 meV is similar to that observed in other proteins (Diehl et al. 1997; Paciaroni et al. 1999a) and it is clearly reminiscent of the boson peak which, as already mentioned, is a common feature of protein and glassy systems. Actually, it results in an excess of vibrational modes over the Debye level. In the Debye approximation, the vibrational density of states $g(v)$, at low frequency, becomes proportional to v^2, and the Bose factor $n(v,T) = [exp(hv/k_BT) - 1]^{-1}$ can be

approximated by $1/v$. Hence, the frequency dependence of the inelastic term in Eq. 1 is cancelled out by giving a constant contribution to the dynamic structure factor. Such an excess of vibrational modes should be also visible in the density of states (Cusack and Doster 1990). Figure 3 shows the density of states, $g(v)$, as calculated by following the method described by Taraskin and Elliott (1997), for the fast hydrated protein sample at 100 K. Actually, up to about 2 meV, $g(v)$ follows the Debye level (see the dashed line in Fig. 3), while at higher energies it exceeds such a level. This is in agreement with what has been found for myoglobin by Cusack and Doster (1990).

The presence of the 1 meV peak is indicative of the occurrence of additional vibrational modes at lower frequencies in sample A with respect to sample B. It should be mentioned that a similar effect was not observed in the H/D-substituted dry myoglobin, where a constant value was observed for such a ratio within all the energy range investigated (Diehl et al. 1997). Such a discrepancy could be attributed to various reasons, such as the different hydration state of the samples, a possible incomplete isotopic substitution of the exchangeable protons in myoglobin, or a peculiar behaviour displayed by the protein under analysis.

To better investigate the two observed peaks, we have calculated the dynamic structure factor in the q and v form. The $S(q,v)$, at fixed q ($q = 1.5$ Å^{-1}) [derived from the $S(\theta,v)$ data as reported in Materials and methods], is shown in Fig. 4 at 100 K for the two samples. Again, a single peak at 3.5 meV is present in the dynamic structure factor of the B sample; besides this peak, another one at a lower energy (1 meV) is visible in the dynamic structure factor of sample A. The inset of Fig. 4 shows a q^2 trend for the intensity of $S(q,v)$ as a function of q; such behaviour provides evidence for the dominant incoherent contribution to
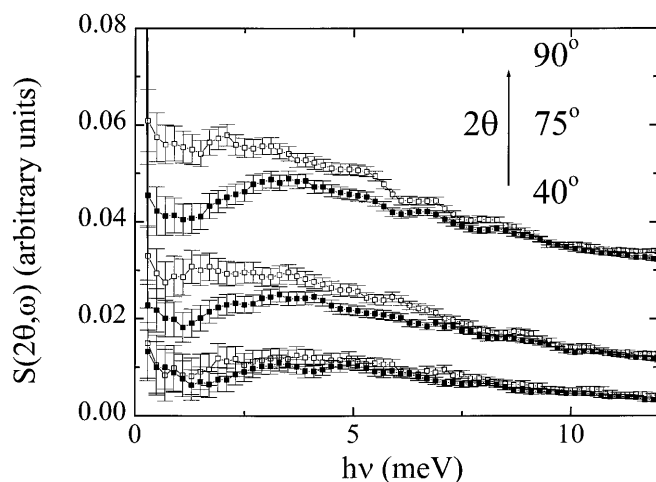

Fig. 2 Dynamic structure factor, $S(2\theta,v)$, as a function of energy at 100 K for three different scattering angle values, of D$_2$O-hydrated PC containing exchangeable and non-exchangeable protein protons (sample A: *empty squares*) and of D$_2$O-hydrated PC containing non-exchangeable protein protons (sample B: *filled squares*)
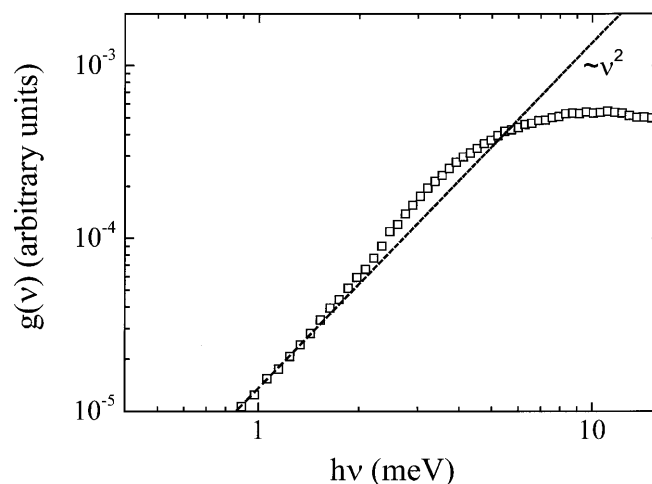

Fig. 3 Vibrational density of states at 100 K, as a function of energy, for D$_2$O-hydrated PC containing exchangeable and non-exchangeable protein protons (sample A). The *dashed line* shows the Debye level: $g(v) \approx v^2$
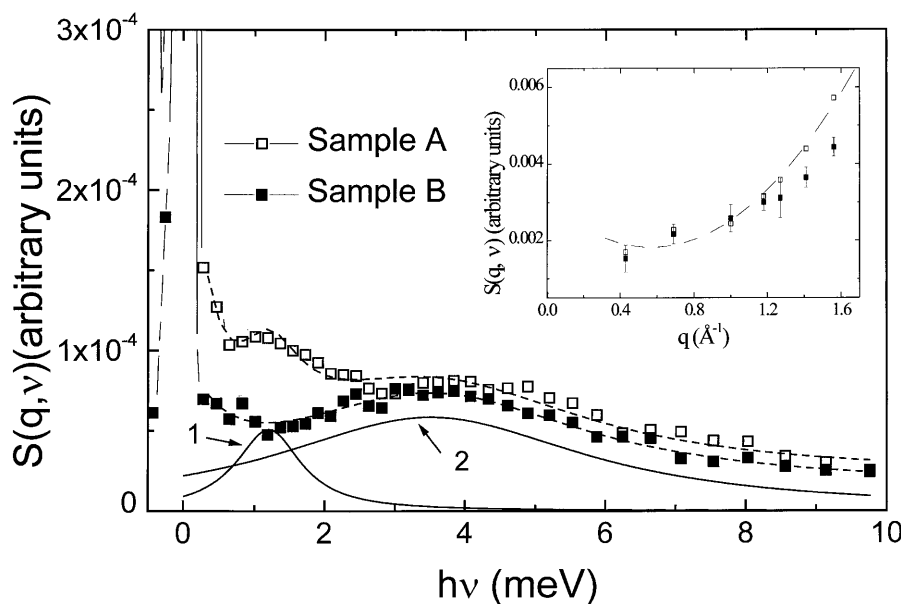
Fig. 4 Dynamic structure factor, $S(q,v)$, as function of energy at a fixed wavevector $q = 1.5$ Å^{-1}, of sample A (*empty squares*) and sample B (*filled squares*) at 100 K. *Curve 1*: Lorentzian lineshape, $A_1/[(E–E_1)^2 + \sigma_1^2]$, with parameters $A_1 = 3.9 \times 10^{-5}$, $E_1 = 1.0$ meV, $\sigma_1 = 0.9$ meV; *curve 2*: Lorentzian lineshape, $A_2/[(E–E_2)^2 + \sigma_2^2]$, with parameters $A_2 = 4.4 \times 10^{-4}$, $E_2 = 3.5$ meV, $\sigma_2 = 2.7$ meV. *Upper dashed line*: fitting curve given by a superposition of curve 1, curve 2 and background. *Lower dashed line*: fitting curve given by a superposition of curve 2 and background. *Inset*: Intensity of the 3.5 meV peak [obtained by integrating the dynamic structure factor $S(q,v)$ between 2.5 and 3.5 meV] as a function q for both the A and B samples. The *dashed line* shows a q^2 fit as derived for sample A data

the inelastic region. In fact, it could be assumed that, in analogy with glasses, a coherent component, due to in-phase modes, should display a characteristic $q^2 S(q)$ dependence which includes sound-wave modes and long-wavelength relaxational modes (Buchenau et al. 1996). The inelastic spectrum of sample B can be fitted by a Lorentzian, $A/[(E–E_0)^2 + \sigma^2]$, centered at $E_0 = 3.5$ meV (see Fig. 4), superimposed on a background (a constant level added to an exponential decay). Phenomenologically, the Lorentzian describes the vibrational contribution to the scattering, at a fixed wavevector q, of an oscillator whose energy is distributed around a characteristic energy E_0. Conversely, the dynamic structure factor of sample A can be fitted (see Fig. 4) by two Lorentzians: one with the same parameters (see the legend of Fig. 4) as that reproducing the spectrum of sample B, and another one centered at 1 meV (see Fig. 4), in agreement with that observed in the inset of Fig. 1. Indeed, the position of the second peak corresponds closely to the energy value at which the maximum of the ratio between the dynamic structure factor of the A and B samples occurs (see inset of Fig. 1). These results provide clear evidence that exchangeable and non-exchangeable protein protons may reveal different vibrational features. Actually, it comes out that the boson peak is essentially due to

non-exchangeable protein protons, while the peak at lower energy can be attributed to the exchangeable ones.

The non-exchangeable protein protons are located in the backbone (about 20%) and in the non-polar side chains (80%). A significant amount of the latter are found in the hydrophobic core of the macromolecule (35%), while the remainder are placed on the external surface (65%). Such an analysis has been performed by the method described by Gerstein and Chothia (1996) applied to the protein data bank PC structure (Guss et al. 1992). It is worth noting that the presence of small hydrophobic clusters, whose structure is highly conserved, at the exterior part of PC has been suggested to play a crucial role in the folding process (Tisi and Evans 1995).

Generally, solvent-exposed non-polar side-chains groups, which are mainly not involved into the H-bond network, are characterized by a quite large mobility. Actually, MD simulation of hydrated PC showed that a large part of the non-polar groups, at the protein/solvent interface, belongs to turns connecting the β-sheets and is characterized by large values of the mean square fluctuations (Ciocchetti et al. 1997). This seems to be indicative that the boson peak does not reflect the specific structural properties of the macromolecular architecture, in agreement with the fact that it has been also found in mixtures of amino acids (Diehl et al. 1997). Instead, in analogy with that suggested for amorphous, disordered systems, the boson peak could be related to the topological disorder of proteins (Elliott 1992; Paciaroni et al. 1999b), which have revealed a marked glassy character (Iben et al. 1989; Fan et al. 1994). Actually, the boson peak might originate from the disorder-induced scattering, i.e. the vibrations at and below such a peak could be trapped as localized vibrations, over an intermediate range scale, coexisting and interacting with sound waves

(Buchenau 1992; Elliott 1992). In this framework, we have previously estimated, by taking into account a reasonable value for the sound velocity, the correlation length corresponding to the boson peak excitations for another copper protein, azurin (Paciaroni et al. 1999b). The results showed that the correlation length spans almost the entire protein dimension, suggesting, on the one hand, a delocalized character for such a mode, and, on the other, the possibility that more than one molecule could be involved in such a process. On such grounds, the shift of the boson peak to lower frequency in dry protein systems (Diehl et al. 1997; Paciaroni et al. 1999b) might be explained in terms of a different packing of the overall structure, leading to a lower value for the sound velocity.

The exchangeable protein protons, still present in sample A but removed in sample B, appear to be responsible for the observed peak 7 at 1 meV. These protons are located in the amide groups of the backbone, where they are generally involved in the intramolecular H-bond network stabilizing the macromolecular structure. On such a basis, it might be hypothesized that the peak at 1 meV in sample A could be related to the fluctuations of the H-bonds. In such a case, a possible dependence on the macromolecular secondary structure should be expected.

It should be remarked that MD simulations of the dynamic structure factor of several proteins have been unable to correctly reproduce the energy peak value of the excess vibrational modes (Steinbach et al. 1991; Paciaroni et al. 1999a). Actually, a lower value (1.3 meV) for such a peak has been found; possible reasons for this discrepancy have been searched for among intermolecular interactions, electrostatic cutoff, and an inaccurate force field (Diehl et al. 1997; Melchionna and Desideri 1999). However, the present experimental results represent a basis to revisit the comparison of MD and neutron scattering results. MD simulation deals with one isolated macromolecule, thus providing single-molecule dynamic properties. Reasonably, in a neutron scattering experiment, we deal with a hydrated powder where the macromolecules can also interact among themselves through the overlapping of their hydration shells. We can therefore speculate about the possibility that the 1 meV peak could be a peculiar property of the single macromolecule and is well reproduced by MD. On the other hand, the boson peak at 3.5 meV, which is not reproduced by MD, may be related to dynamic aspects involving several macromolecules.

Finally, it should not escape our attention that the presence of an excess of scattering peaking at 3.5 meV has been observed experimentally also in the hydration water of PC (Paciaroni et al. 1999b). Therefore, in the light of the present results, it is tempting to wonder if the boson peak is, in some way, related to the dynamic coupling between the hydration water and the protein macromolecules (Doster et al. 1986; Arcangeli et al. 1998; Viktup et al. 2000).

Conclusions

A controlled H/D isotopic exchange has allowed us to provide different low-frequency features in the neutron scattering dynamic structure factor of PC. The observed excess of vibrational modes peaking at about 3.5 meV, common to other macromolecular systems and reminiscent of the boson peak found in amorphous systems, is found to be due to the non-exchangeable protein protons. It should be connected to the topological disorder and might involve intermolecular interactions. The peak at about 1 meV, usually reproduced by MD simulations, is essentially due to exchangeable protein protons and might be connected to the H-bond fluctuations.

These results open a new perspective in the analysis of the dynamic features of proteins as investigated by neutron scattering.

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