Dynamics of Different Hydrogen Classes in β-lactoglobulin: A Quasielastic Neutron Scattering Investigation

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The pico- and nanosecond dynamics of the globular protein β-lactoglobulin were investigated by means of quasielastic neutron scattering. To discriminate the possibly different dynamical behaviors of internal and external protein residues, the spectra of properly H/D exchanged samples, both dry and hydrated with D_2O, were measured. In dry samples, inner and outer protein regions are found to provide equal contributions to the quasielastic scattering intensities, over the whole explored temporal window. On the contrary, their dynamical behavior becomes differentiated in the hydrated samples. In particular, in the nanosecond time scale, internal residues appear to be characterized by a higher quasielastic scattering intensity than that of the external ones. A reversed behavior is instead observed in the picosecond time range, where the highest contribution arises from external amino acidic groups. These results provide new experimental evidence of a diversified dynamical behavior displayed by internal and external protein regions. In addition, they point out that hydration water affects the dynamics of the whole biomolecule, rather than that of the solvent-exposed regions only.

Introduction

The dynamical behavior of proteins is recognized to be of outstanding importance to their biological functionality. Molecular motions are indeed known to confer to proteins the conformational flexibility required for their function.[1,2]

As a consequence of the structural complexity of biomolecules, their dynamical properties are highly diversified. The long amino acidic chain of proteins and their large number of polypetide side groups yield a rich variety of motions, ranging from fast local vibrations of small atomic groups to slow collective movements of large domains.[2] Such a dynamical richness results in a huge distribution of motional characteristic times, which range from 10^{-14} s to 10^{1} s, corresponding to energies from 10^{2} meV to 10^{-13} meV.[2] Within this wide dynamical window, pico- and nanosecond movements give particularly important contributions.[2,3] Although, on one hand, motions of such time scales are easily accessible with the up-to-date experimental and computational resources, on the other hand, it is often suggested that these movements could play an important role in triggering and driving the conformational transitions required by the biological functionality.[3−8]

In the past, protein dynamics have been investigated with a large number of techniques, such as calorimetric measurements,[9] Mössbauer[10] and infrared spectroscopy,[7] NMR relaxation methods,[11−13] molecular dynamics (MD) simulations,[14] X-ray crystallography,[15] and neutron scattering.[6,16,17] Early studies provided a general characterization of the molecular motions, focusing on the effects of temperature and hydration level.[3,8,11,15−23] Indeed, these two parameters are of fundamental importance for the protein dynamics, as both are known to affect the onset of large-amplitude anharmonic motions, which are likely to be involved in the biological function.

With this respect, the role of temperature is revealed by the observation that, in hydrated proteins, anharmonic large-amplitude motions are almost frozen below $T_d = 200$ K. On the contrary, above T_d, the motional amplitudes increase with increasing temperature, and, at physiological conditions ($T \cong 300$ K), the relevant movements become fully active.[8,13,16,19,20] Concerning instead the role of the hydration level, even at physiological temperatures functional motions are strongly inhibited in dry proteins. The addition of hydration water gradually allows the onset of the anharmonic large-amplitude movements, which reach their full activity at a hydration level of $h = 0.38$ g of H_2O per gram of protein.[23]

Hydration water is therefore of utmost importance for the protein dynamics, as it induces the molecular mobility necessary to the biological function. In this context, the question arose of determining which protein structural elements are mostly affected by the presence of the solvent, and are thus mainly responsible for the onset of the functional motions. Recent studies on various proteins attempted to clarify these aspects. On the basis of common theoretical models,[2] the protein atomic groups which could give origin to pico- and nanosecond motions have been proposed.[18] Some examples include the three-site jump diffusion of hydrogens belonging to methyl groups, the two-site jump diffusion of protons involved in hydrogen bonds, and the diffusive and relaxational movements of various protein subdomains, such as reorientational diffusion of peptide side-chains or relative motions of molecular subunits.[18] By means of quasielastic neutron scattering, these movements have been experimentally classified, according to either their correlation times[18,19] or their amplitudes.[4] The influence of hydration on the various motional components has also been analyzed and it has been commonly assumed that water mostly affects the mobility of surface protein residues.[19,21] Nonetheless, this idea has been reconsidered by some studies, which showed that the protein flexibility is globally increased by the presence of the

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solvent, as the dynamics of internal residues is also strongly enhanced.[11,22,24,25]

Concerning the role played by internal and external protein shells in particular, their dynamical contributions have recently been analyzed in more detail by means of site-resolution techniques, such as NMR[11] or MD simulations.[26,27] With this respect, the investigation on the behavior of exchangeable and nonexchangeable protein hydrogen atoms can be helpful. Indeed, the structure of some globular biomolecules is such that the large majority of exchangeable hydrogens is located on the protein surface, whereas nonexchangeable ones are concentrated in the protein core. Thus, by means of an appropriate contrast technique, the study and comparison of suitably H/D exchanged samples can highlight possible differences between the dynamics of surface and core residues. Few experiments of this kind have been actually carried out.[28,29]

Neutron scattering is an efficient contrast technique to discriminate between signals arising from hydrogen and deuterium atoms.[31,32] Thus, we have recently performed a neutron scattering experiment on β-lactoglobulin (βLG), aimed at pointing out, through an H/D-exchange-based analysis, the inelastic response of exchangeable and nonexchangeable protons between 1 and 10 meV.[25] In this energy window, protein spectra are known to display an anomalous vibrational bump, which resembles the boson peak presented by glasses. We found out that, in dry βLG, exchangeable hydrogen atoms give the anomalous peak a lower-frequency contribution (2.2 meV) than nonexchangeable ones (3 meV). Moreover, exchangeable protons turned out to provide a stronger inelastic scattering intensity than nonexchangeable ones. An extension of this selective study to the quasielastic spectral features might actually be interesting, as, on a theoretical ground, a direct connection has been suggested between the boson peak, observed at low temperature, and the temperature-increasing quasielastic scattering.[30] In addition, the analysis of the quasielastic shape at physiological temperatures, rather than of the low-temperature inelastic response, can provide more direct information on the large-amplitude anharmonic motions of functional relevance.

In the present paper, we extend our previous H/D-exchange-based analysis of βLG to lower energy regions, namely in the ranges $1-15\ \mu$eV and $0.1-1$ meV, by studying the quasielastic response of the protein with two neutron spectrometers of different energy resolution and transfer range. Indeed, such energy windows correspond to the nano- and picosecond time scales, which are of particular interest to the study of protein motions.[3]

Materials and Methods

1. Sample Preparation. β-lactoglobulin[12,33−35] (βLG) is a β-barrel globular protein, belonging to the lipocalin family, which can be extracted from milk whey and is thought to be involved in the transport of ligands, such as retinol or fatty acids. βLG is composed by a sequence of 162 amino acids, corresponding to a molecular weight of 18 155 Da, and its chemical composition is $C_{820}H_{1308}N_{206}O_{252}S_9$. The 1308 protein protons can be classified in exchangeable and nonexchangeable, according to their chemical bond to the protein sequence.[36,37] Exchangeable hydrogens sum up to 20% of the total amount of the βLG protons (240 out of 1308) and are mainly located ($\sim$90%) on the outer shells of the protein structure. On the other hand, nonexchangeable hydrogens are mainly situated in the protein interior.[12]

The four samples described in Table 1 were prepared by suitable H/D exchange procedures. The details of the sample preparation are described in the following. All of our βLG powders were purchased by SIGMA Aldrich. Sample D was prepared by simply dehydrating 300 mg of protein as commercially obtained. The sample was first lyophilized and then desiccated under vacuum in the presence of P_2O_5 for 2 days, achieving a final hydration level lower than $h = 0.05$ g of water per gram of protein. As it did not undergo any exchange process, this sample contains both exchangeable and nonexchangeable protons. In sample DD, all exchangeable protons are wanted to be substituted with deuterium atoms. Therefore, 370 mg of βLG powder were first dissolved in 15 mL of D_2O for 1 week and then lyophilized. These two steps were repeated three times, to achieve a complete proton exchange. Finally, the exchanged protein was desiccated under vacuum with P_2O_5 for 2 days, achieving a final hydration level lower than $h = 0.05$ g of water per gram of protein. The two hydrated samples, sample H and sample HD, were obtained by hydrating with D_2O two further dry and dry deuterated powders, prepared by the previously described procedure. The hydration process was carried out in a chamber under vacuum in the presence of a saturated KCl and heavy water solution, achieving a final hydration level of $h = 0.4$ g of D_2O per gram of protein. The hydration state of the four samples was carefully checked, both before and after the neutron scattering experiments, and was found to remain unchanged.

TABLE 1: Measured β-lactoglobulin Samples

sample	Hydrogen content
dry (D)	containing all protein hydrogen atoms
dry deuterated (DD)	containing only nonexchangeable protein hydrogen atoms, with exchangeable ones substituted by deuterium atoms
D_2O−Hydrated (H)	containing all protein hydrogen atoms, hydrated at $h = 0.4$g of D_2O pergram of protein
D_2O−Hydrated deuterated (HD)	containing only nonexchangeable protein hydrogen atoms, with exchangeable ones substituted by deuterium atoms, hydrated at $h = 0.4$g of D_2O per gram of protein

2. Incoherent Scattering Function. In a βLG molecule, as in the majority of the biological systems, hydrogens constitute about 50% of the total number of atoms. It is well-known that the neutron−hydrogen cross-section (81.7 barns) is mainly incoherent ($\sim$98%) and is about 1 order of magnitude larger than the neutron cross-sections of other protein atoms. This disproportion holds, in particular, for the neutron-deuterium cross-section (7.6 barns).[31,32] These facts have two main implications. First, in a neutron scattering experiment on a biological sample, the large majority of the detected signal is due to hydrogen atoms. Thus, in the treatment of the measured spectra, the coherent contribution is usually neglected. Indeed, in our previous neutron scattering study of βLG, the q dependence of the collected spectra was carefully checked and revealed that the coherent component is largely negligible in all our samples.[25] Second, if the spectra of a fully hydrogenated sample and of a partially deuterated sample are compared to each other, in the latter, the signal arising from deuterium atoms can be neglected with respect to that due to the remaining hydrogen atoms; thus, the differences between the spectra of the two samples provide information about the H/D substituted group of hydrogen atoms. This contrast technique is therefore a valuable method to highlight and compare the dynamics of different protein regions, as in the present case.

In an inelastic neutron scattering experiment on an isotropic sample, the measured quantity, providing physical information about the studied system, is the scattering function $S(q,\omega)$; here, q is the modulus of the momentum transfer and ω is the energy transfer, both in units of the reduced Planck constant p. In the one-phonon approximation, the incoherent scattering function, at a given temperature T, can be written as the sum of three terms

$$S(q,\omega) = e^{-2W(q,T)}\{A_0(q)\cdot\delta(\omega) + [1 - A_0(q)]\cdot S_{QE}(q,\omega)\} + S_{INEL}(q,\omega) \quad (1)$$

where $e^{-2W(q,T)}$ is the Debye−Waller factor, $A_0(q)$ is the elastic incoherent structure factor, $\delta(\omega)$ is the Dirac delta function, $S_{QE}(q,\omega)$ is the quasielastic incoherent dynamic structure factor and $S_{INEL}(q,\omega)$ is the inelastic incoherent scattering function.[32] The first term takes into account for the elastic response of the system, while the last term describes its vibrational behavior. The second term, appearing as a broadening of the elastic peak, gives information about the protein diffusive and relaxational movements, which we are going to deal with. Due to the high complexity of the protein structure, many different kinds of motions can give rise to quasielastic scattering, as already mentioned in the Introduction. Indeed, the movements of the highly diversified molecular subunits produce quite different quasielastic contributions. Moreover, even identical subunits may experience different local environments and thus move in different ways. It is therefore very difficult to find an exact theoretical function to describe the quasielastic scattering term. It is more useful to model $S_{QE}(q,\omega)$ through a phenomenological function, such as a sum of Lorentzians[19,38]

$$[1 - A_0(q)]\cdot S_{QE}(q,\omega) = \sum_n \mathrm{QISF}_n(q)\cdot L_n(\sigma_n,\omega) \quad (2)$$

where $L_n(\sigma_n,\omega)$ is the n-th Lorentzian, σ_n is its full width at half-maximum (fwhm), the inverse of which provides an estimate of the characteristic time scale of the corresponding motion, and $\mathrm{QISF}_n(q)$ is the quasielastic incoherent structure factor, which provides the energy integral of the n-th quasielastic component and quantifies the degree of activity of the relevant motion. Due to the large variety of quasielastic components in protein spectra, every Lorentzian represents, rather than a single kind of movement, a broad, almost continuous distribution of motions, each characterized by its own correlation time and related line width. Thus, in this context, σ_n should be regarded as an "effective" line width, whereas $QISF_n(q)$ should be considered as a quantitative measure of the activated dynamical processes which L_n represents.

In real experiments, eqs *1* and *2* must be convoluted with the instrumental energy resolution function, which as a finite characteristic line width. The inverse of such line width determines the time scale of the slowest observable motion. Slower motions contribute to the elastic region of the spectrum and are not observable as a quasielastic signal. On the other hand, the inverse of the maximum achievable energy transfer defines the correlation time of the fastest observable motions. Therefore, both the energy resolution and the energy transfer range determine which portion of the broad distribution of motions is picked up by the measurement (see the following subsections **3** and **4**, for the explicit definition of the presently explored time-windows).

The aim of this paper is to single out the contributions to the scattering function arising from exchangeable and nonexchangeable protein hydrogens. With this respect, let $S_e(q,\omega)$ and $S_{ne}(q,\omega)$ be the average scattering functions of an exchangeable and a nonexchangeable proton, respectively. In terms of $S_e(q,\omega)$ and $S_{ne}(q,\omega)$, the scattering function of a generic hydrogen atom can be written

$$S_{all}(q,\omega) = \frac{N_e}{N_e + N_{ne}} S_e(q,\omega) + \frac{N_{ne}}{N_e + N_{ne}} S_{ne}(q,\omega) \quad (3)$$

where N_e and N_{ne} are respectively the number of exchangeable and nonexchangeable protons in a molecule. In eq *3*, the three scattering functions are all normalized to a single hydrogen atom of the corresponding type. To put this into perspective, it is worth of notice that the experimental scattering functions are also normalized to a single hydrogen atom, as explained in the following subsections **3** and **4**. In particular, the scattering function of the average nonexchangeable proton, $S_{ne}(q,\omega)$, is provided by the measured spectra of the deuterated samples, DD and HD. Meanwhile, a measure of $S_{all}(q,\omega)$ is given by the spectra of samples D and H. Therefore, the scattering function of the sole exchangeable hydrogens $S_e(q,\omega)$ can be obtained from our experimental data, by means of eq *3*.

3. Neutron Scattering Experiment. To measure the quasielastic spectra of our samples in the μeV range, the backscattering spectrometer IN16 at the Institut Laue-Langevin (ILL) in Grenoble (France) was employed. An incoming neutron wavelength of 6.27 Å was used, achieving an elastic q-range from 0.02 $Å^{-1}$ to 2 $Å^{-1}$, an exchanged energy range from −15 to 15 μeV and an elastic energy resolution of 0.9 μeV. These energy transfer range and resolution allow to observe motions with characteristic times between about 100 ps and 5 ns.

The samples were placed in standard slab-shaped aluminum cells, bathed in the neutron flux at an angle of 45° with respect to the incident beam. The quasielastic spectra of the two dry samples (D and DD) were measured at 300 K, the spectrum of the D_2O-hydrated deuterated sample (HD) was measured at 250 and 300 K, the spectrum of the D_2O-hydrated sample (H) was measured at 100, 250 and 300 K.

The quasielastic spectra in the meV range were measured by means of the high-flux time-of-flight spectrometer IN6, at ILL in Grenoble (France). An incident wavelength of 5.1 Å was employed, achieving a q-range from 0.2 $Å^{-1}$ to 2.0 $Å^{-1}$, an accessible energy transfer range from −1.9 meV to $2k_BT$ and an elastic energy resolution of 90 μeV. These energy ranges and resolutions allow us to observe motions with characteristic times between about 0.1 and 50 ps.

The four samples were held in standard slab-shaped aluminum cells, placed into the neutron flux with an angle of 135° with respect to the incident beam. Spectra were collected at three temperatures for each sample, namely at 100, 220, and 300 K, for a period of time ranging from 2 to 6 h, depending on the temperature.

Standard corrections and normalizations were performed in a similar way in both the μeV and the *m*eV range. Spectra from an empty can and a cadmium absorber were collected at 300 K to take into account for the various scattering contributions due to the sample environment. A vanadium standard scan was performed to measure the resolution function and to take into account for the different detector efficiencies. After having checked and removed the detectors showing a too-high electronic noise, the data reductions were performed using standard ILL programs, which correct spectra for incident flux, cell and environment scattering, detector efficiency and self-absorption. Because all samples have transmissions of about 92%, multiple scattering and multiphonon corrections were not applied. To

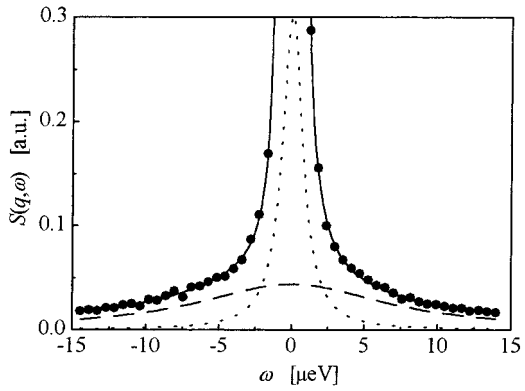

Figure 1. Fit of the μeV spectrum of sample D. Full circles: experimental data points; the experimental error bars are smaller than the plotted data points. Full line: best fit function. Dotted line: slow Lorentzian component. Dashed line: fast Lorentzian component. The energy transfer ω is in units of the reduced Planck constant $\hbar$; this notation will be adopted for all the following figures.

TABLE 2: Fit Results for SAmples INvestigated at 300K (See Text)

spectrometer	sample	$QISF_1$[a]	σ_1 (fwhm)[b]	$QISF_2$[a]	σ_2 (fwhm)[b]
	D	0.079 ± 0.009	3.3 ± 0.4	0.097 ± 0.009	33 ± 2
IN16	H	0.14 ± 0.03	3.9 ± 0.4	0.234 ± 0.007	32 ± 2
	DD	0.09 ± 0.04	8 ± 4	0.06 ± 0.04	40 ± 30
	HD	0.15 ± 0.04	4.0 ± 0.4	0.275 ± 0.007	30 ± 2
	D	0.118 ± 0.005	0.20 ± 0.01	0.024 ± 0.002	2.8 ± 0.4
IN6	H	0.155 ± 0.002	0.26 ± 0.06	0.0555 ± 0.0009	2.8 ± 0.4
	DD	0.110 ± 0.008	0.20 ± 0.02	0.025 ± 0.005	2.8 ± 1.4
	HD	0.153 ± 0.003	0.26 ± 0.04	0.044 ± 0.002	2.8 ± 0.6

[a] The quasielastic structure factors are normalized to satisfy the condition $A_0 + QISF_1 + QISF_2 = 1$. [b] Measured in μeV for IN16 and in meV for IN6.

improve the data statistics, all of the detectors were binned together, obtaining an average elastic q of 1.5 $Å^{-1}$ on IN16 and 1.2 $Å^{-1}$ on IN16. The corrected spectra were finally symmetrized by the detailed balance factor and normalized in order to have the same energy integral.

Results

In agreement with the previous section (see eq 2), and as commonly found in the literature,[18−21,38] the measured quasielastic spectra were analyzed by fitting them with a sum of Lorentzians convoluted with the instrumental resolution function. Two Lorentzian components were found to adequately reproduce all the spectra, in both the μeV and meV ranges, whereas addition of further components did not significantly improve the fit quality. Figure 1 shows, for instance, the best fit function of the μeV spectrum of sample D, together with the two Lorentzian components. All of the protein motions contributing to the quasielastic signal are thus phenomenologically separated into a "slower" and a "faster" class, according to the correlation times derived by the two Lorentzian line widths. The obtained best fit parameters are reported in Table 2 and consist in the quasielastic incoherent scattering factors, $QISF_{1(2)}$, and the full widths at half-maximum, $\sigma_{1(2)}$, where the subscripts 1 and 2 denote the slow and the fast component, respectively. In the following, the μeV and the meV measured spectra are reported and commented upon, together with the features revealed by the fitting procedure.

1. The μeV Energy Range. Figure 2 shows the temperature dependence of the quasielastic spectra of βLG, in the μeV range explored by the spectrometer IN16. In this energy region,

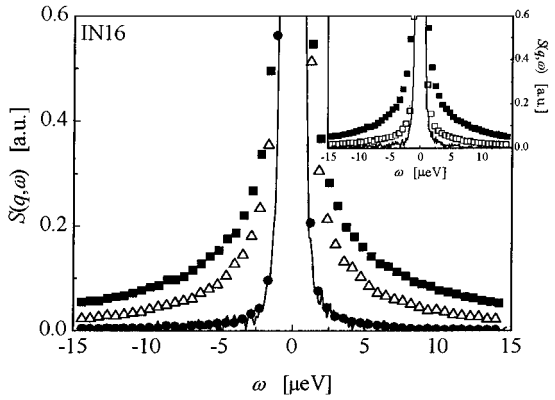

Figure 2. Temperature dependence of quasielastic scattering in βLG. The μeV spectrum of sample H is plotted at 300, 250, and 100 K, from top to bottom. Experimental error bars are smaller than the plotted data points. The full line is the vanadium spectrum, which provides the instrumental resolution function of IN16. *Inset.* The μeV spectra, at 300 K, of sample H (full squares) and sample D (open squares) show the hydration dependence of quasielastic scattering in βLG. The full line is the vanadium spectrum.

corresponding to nanosecond motions, inelastic protein features are not observable, and thus, these spectra can be accounted for by the first two terms of eq 1 only. The scattering functions shown in Figure 2 belong to the D_2O-hydrated sample (H), at the three temperatures of 100, 250, and 300 K, from bottom to top. The full line is the vanadium spectrum and represents the instrumental resolution function. At 100 K, the sample spectrum overlaps exactly with the resolution, thus indicating that the quasielastic scattering is negligible at this temperature. Therefore, the possible protein motions are too slow to be observed with the present, or a lower, energy resolution. With increasing temperature, as displayed by the 250 and 300 K spectra, quasielastic scattering becomes more and more intense, thus witnessing the onset of protein nanosecond motions. The effect of hydration on the 300 K spectrum is shown in the inset of Figure 2. In agreement with well-known results, the hydrated sample displays a more intense quasielastic scattering than the dry one; highlighting thus the fundamental role of hydration water in the activation of further protein diffusive motions and conformational transitions.

Further information on the temperature and hydration level dependence of the quasielastic spectra is provided by the fit results in Table 2. In the energy range of IN16, both the slow (1) and the fast (2) Lorentzian components display line widths which resulted to be equal in all the four samples. Such line widths yield correlation times of about 1 ns and 150 ps, respectively. The quasielastic incoherent structure factors, on the contrary, were found to vary with both temperature and hydration level. In particular, in the hydrated samples, $QISF_1$ and $QISF_2$ are strongly enhanced with respect to the dry samples. In light of the these results, it can be inferred that hydration water does not affect the motional correlation times, but it rather triggers the onset of additional movements, which seem to be unlocked by the presence of the solvent within a sort of "on−off" mechanism. Upon changing the temperature, the Lorentzian line widths were again found to remain constant, whereas the quasielastic structure factors were found to vary (the relevant fit results are not reported). In particular, a temperature decrease from 300 to 250 K was found to approximately halve the integrated intensities QISF. At 100 K, as already underlined, the quasielastic signal becomes negligible.

To highlight the possible quasielastic differences between exchangeable and nonexchangeable hydrogen atoms, only the

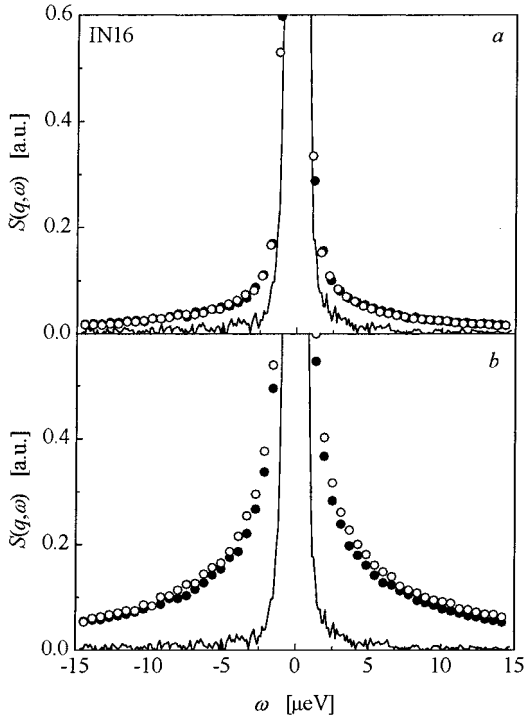

Figure 3. (a) Quasielastic spectra of sample D (full circles) and sample DD (open circles), in the μeV energy range. Experimental error bars are smaller than the plotted data points. The full line is the resolution function, provided by the vanadium spectrum. The elastic peak intensity of both spectra is 9.1 au. (b) Quasielastic spectra of sample H (full circles) and of sample HD (open circles), in the μeV energy range. Experimental error bars are smaller than the plotted data points. The full line is the resolution function of IN16. The elastic peak intensities of the two spectra are 7.9 and 7.6 au, respectively.

spectra at 300 K will be considered in the following. Indeed, at this temperature, which is also closer to physiological conditions, quasielastic scattering is more intense.

Figure 3*a* shows the μeV spectra of the dry (D) and dry deuterated (DD) samples, at 300 K. The resolution function is also plotted for comparison. Within the experimental error bars, which are smaller than the dimensions of the plotted data points, the two dry samples display the same normalized quasielastic scattering intensity. The experimental scattering functions of sample D and sample DD correspond to a measure of $S_{all}(q,\omega)$ and $S_{ne}(q,\omega)$, respectively. If, according to the experimental data, the equality $S_{all}(q,\omega) = S_{ne}(q,\omega)$ is introduced in eq 3, it turns out that $S_e(q,\omega) = S_{ne}(q,\omega)$.

The effect of hydration, on the two above-mentioned hydrogen classes, is shown in Figure 3b, where it is observed that, in the whole explored μeV range, the quasielastic scattering intensity of sample HD is systematically higher than that of sample H. By referring again to eq 3, the experimentally observed higher intensity of $S_{ne}(q,\omega)$ with respect to $S_{all}(q,\omega)$ implies that $S_{ne}(q,\omega) > S_e(q,\omega)$.

2. meV Energy Range. To study the behavior of quasielastic scattering in the meV energy region of IN6, an additional problem must first be faced. In Figure 4, it can be observed that, besides quasielastic scattering, the meV spectrum of βLG displays a broad inelastic peak at about 3.5 meV, therefore the third term of eq 1 can no more be neglected. The inelastic bump, commonly termed "boson peak,"[5,17,20,28,29,39] is clearly visible at 100 K, whereas at 300 K, it is masked by the rising quasielastic band.[25,40] To study the 300 K quasielastic scattering only, it is necessary to properly get rid of the underlying inelastic contribution. This could be achieved by rescaling the 100 K

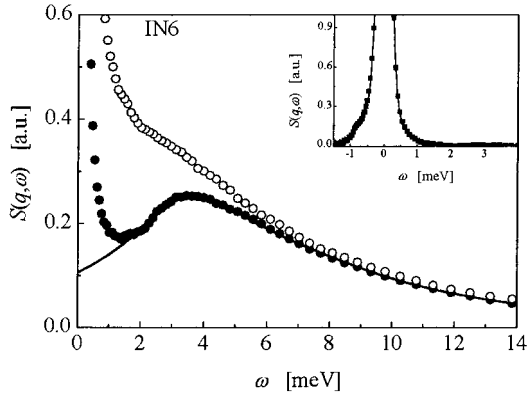

Figure 4. Inelastic spectrum of sample H, in the meV energy range, at 100 (full circles) and 300 K (open circles). The 100 K spectrum has been rescaled at 300 K by the Bose factor. The full line is the best fit of the inelastic peak. *Inset.* Full squares: resolution function of IN6, obtained from the vanadium spectrum. Full line: difference between the whole 100 K spectrum of sample H and its inelastic peak fit. The comparison of the two plotted curves provides a check on the procedure employed to get rid of the inelastic component (see text for details).

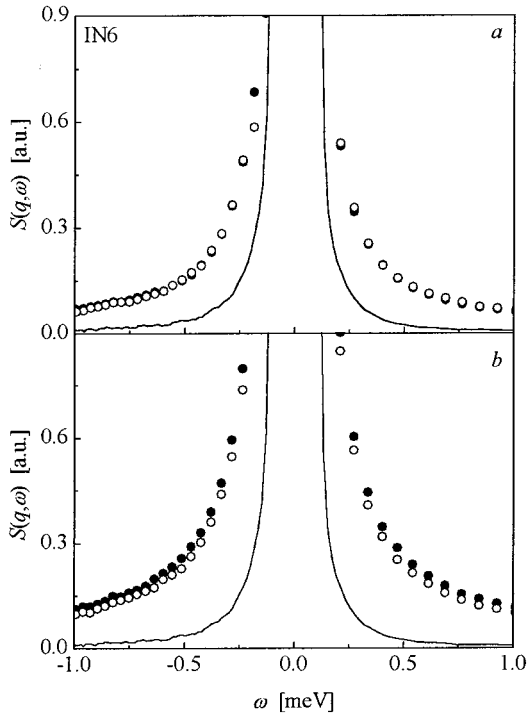

Figure 5. (a) Quasielastic spectra of sample D (full circles) and of sample DD (open circles), in the meV energy range. Experimental error bars are smaller than the plotted data points. The full line is the resolution function of IN6. The elastic peak intensity of both spectra is 89 au. (b) Quasielastic spectra of sample H (full circles) and of sample HD (open circles), in the meV energy range. Experimental error bars are smaller than the plotted data points. The full line is the resolution function of IN6. The elastic peak intensities of the two spectra are 75 and 77 au, respectively.

spectrum at 300 K, according to the Bose factor, and then subtracting such rescaled spectrum to the 300 K spectrum. To this end, it should be assumed that quasielastic scattering is negligible at 100 K, and thus, at this temperature, the scattering function provides the sole inelastic component.[17,28,40] Indeed, in Figure 2, the overlap of the 100 K spectrum with the resolution function supports this assumption. In carrying out such outlined procedure, however, it should be taken into account that a possible missubtraction of the intense elastic peak could considerably spoil the resulting quasielastic shape. To

avoid this, an alternative procedure was followed. In previous analyses,[25,29] the vibrational bump was fitted with the sum of two Lorentzians. The same method has been herewith employed to model the boson peak rescaled at 300 K, for energies greater than 1.5 meV. The obtained best fit function, plotted as a full line in Figure 4, was extended to the whole energy range and then subtracted to the 300 K spectrum. The result is a good approximation of the sole quasielastic scattering at 300 K. As a check on the reliability of this procedure, the best fit function of the inelastic bump was rescaled again at 100 K and then subtracted to the entire 100 K spectrum. If, at this low temperature, quasielastic scattering were really absent, then the resulting curve should contain the elastic contribution only. Indeed, the good overlap we found between the resulting curve and the resolution function (see the inset of Figure 4) supports both the reliability of the subtraction procedure and the assumption that the quasielastic scattering is negligible at 100 K.

Figure 5, parts a and b, shows the 300 K quasielastic spectra, obtained from the above data treatment, for the dry and hydrated samples, respectively. These spectra were analyzed with the two-component fitting procedure outlined above (see Table 2). As in the μeV energy range, increasing both the temperature and the hydration level results in an increase of the integrated intensities *QISF*, with the line widths σ remaining unaltered. Such line widths yield correlation times of about 20 ps for the slow component and 1 ps for the fast component.

Concerning possible differences in the dynamical behavior of exchangeable and nonexchangeable hydrogens, the two dry samples display the same quasielastic scattering intensity, as also found in the μeV range. Conversely, in the hydrated samples, some differences are observed. Sample H, containing both exchangeable and nonexchangeable hydrogen atoms, displays a systematically higher quasielastic scattering intensity. It should be noticed that such observation is opposite to what found in the μeV range.

Discussion

The temperature and hydration level dependence of protein quasielastic spectra has been widely studied on many different kinds of protein systems. The findings on βLG herewith reported are in substantial agreement with results existing in the literature.[3,8,11,15−23] Therefore, they will not be further discussed. Rather, attention will be paid to the comparison between the behavior of exchangeable and nonexchangeable hydrogen atoms, which bear witness to the dynamics of surface and core protein residues, respectively.

The reported results indicate that these two protein regions, in dry βLG, are characterized by a similar mobility in both of the studied energy ranges. Protein large-amplitude movements, suggested to be involved in the biological functionality, are known to be strongly inhibited by a low hydration level. This is not only witnessed by the low quasielastic intensity of the dry samples with respect to the hydrated ones (see the inset of Figure 2), but it is also complemented by the new finding that both the inner and the outer protein structural elements are prevented to the same extent from performing large-amplitude movements.

The results concerning the hydrated samples provide novel information about the solvent effect on the protein dynamics. Actually, both surface and core amino acidic groups display an increased mobility with increasing hydration level, on both the explored time scales. Therefore, the gain of mobility of external residues, which are in close contact with water, seems to propagate throughout the protein, thus unlocking the movements of internal groups also. Such a finding strengthens the hypothesis that the flexibility of the whole molecule is affected by the solvent,[11,22,24,25] meanwhile contradicts the idea that water mainly acts on solvent-exposed protein regions.[19,21] As concerns βLG in particular, this result is in agreement with some indications obtained from its inelastic spectrum.[25] It is known that the so-called boson peak, characterizing protein vibrational spectra, shifts toward higher frequencies with increasing hydration level.[8,20,28,40] In our previous inelastic neutron scattering investigation of βLG,[25] both internal and external hydrogen atoms resulted to contribute to the boson peak. In addition, both contributions showed a hydration-dependent frequency shift. Thus, also concerning vibrational motions, water affects both inner and outer protein regions.

More detailed information about the influence of hydration water on the protein dynamics is obtained from the intensity differences observed between the two hydrogen classes in the wet samples. Our results show that the solvent-induced increase of mobility is not homogeneous, with respect to both the different location of the protein structural elements and the different energy windows. In addition, the higher mobility exhibited in the meV range by surface groups is in agreement with the vibrational behavior revealed by the inelastic scattering experiment cited above.[25] In that case, besides results concerning the boson peak frequency, external protons were found to contribute to the vibrational bump with a higher scattering intensity than internal ones, thus indicating that the higher mobility of surface residues concerns vibrational motions also.

From a theoretical point of view, the observation that the major scattering contribution arises from different groups of atoms in different energy windows is consistent with the sum rule the incoherent scattering function must satisfy, i.e., $\int_{-\infty}^{\infty+} S(q,\omega)\, d\omega = 1$. As a consequence of this property of $S(q,\omega)$, in the meV spectra of IN6, the intensity excess displayed by surface residues in the inelastic and quasielastic region should be compensated for by a lack of intensity in the elastic region. Indeed, the elastic peak of sample H resulted to have a lower intensity with respect to sample HD, by a factor of about 2%. This means that, in the energy region falling inside the resolution of IN6, internal groups, rather than external ones, provide the strongest scattering contribution. Such a contribution should be revealed as quasielastic scattering by a spectrometer of higher resolution, indeed consistent with what was observed in the μeV spectra of IN16.

On quantitative grounds, the observed intensity difference between deuterated and nondeuterated samples can be supported by the results of the applied fitting procedure. Let us first consider the μeV range, explored by IN16. From the comparison of the fit results for samples H and HD, it turns out that only the parameter $QISF_2$ is significantly different, displaying a higher value for sample HD. Thus, the observed extraintensity of core residues can be addressed to an enhancement of the fast component only. The line width of this component, $\sigma_2 = (30 \pm 2)$ μeV, is narrower than the 90 μeV resolution of IN6, thus explaining why the extraintensity of sample HD is observed with IN16, but not with IN6, which indeed reveals a reversed behavior. The fitting procedure on the IN6 spectra supports this latter finding and reveals that the higher intensity of sample H is due to an increase of the structure factor $QISF_2$ of the fast component only. The line width $\sigma_2 = (2.8 \pm 0.4)$ meV of this component indicates that such an effect arises from movements with relaxational times of about 1 ps.

To give an interpretation of these opposite effects of water in the two explored energy windows, the involvement of the hydrogen bond network, which is established between the hydration shell and the solvent-exposed protein residues, can be suggested. Indeed, it is well-known that such network is an element of fundamental importance in both the stabilization of the protein structure and the sustaining, through hydrogen bond breaking and re-forming dynamics, of the large-amplitude molecular movements.[2,20,24,41] In particular, the typical values of hydrogen bond lifetimes usually range from 0.1 to 10 ps.[18,20] The corresponding energies are such that hydrogen bond-breaking and re-forming dynamics mainly occurs in the meV scale. Therefore, it can be reasonably hypothesized that molecular motions of such energies, of both diffusive and vibrational nature, will be sustained and enhanced by the presence of the hydration shell to a greater extent than motions of different energies. In support of this hypothesis, the correlation time obtained by the line width σ_2 of the fast meV component matches very well the characteristic lifetime of H-bonds. On the contrary, the slower μeV motions, whose correlation times are much longer than those typical of hydrogen bond dynamics, would be less supported by the hydrogen bond network. In a simplified classical picture, it can be thought, for instance, that a protein lateral chain, stabilized by a local fluctuating hydrogen bond network, can more likely move from one of its possible positions to another, if such transition occurs within the time employed by the H-bonds to break and re-form the stabilizing network. On the contrary, if the transition takes longer times, then the side chain will have a higher probability to be relocked by the H-bonds into its initial position, before its movement is carried out.

Conclusion

The results herewith reported point out a different quasielastic scattering behavior of internal and external amino acidic groups in βLG, and provide novel information about the influence that hydration water exerts on the protein dynamics, in the pico- and nanosecond temporal windows.

We found out that, rather than affecting the mobility of surface residues only (as it would be intuitively expected), hydration water strongly enhances the movements of internal atomic groups also, in agreement with some recent studies.[11,22,24,25] In addition, a more detailed analysis revealed that surface protein motions occurring within picosecond time scales are enhanced by the solvent more than those taking place within the nanosecond temporal window.

Such results should be considered in connection with the role of the protein-water hydrogen bond network. We hypothesize that the H-bond network dynamics supports picosecond surface motions, likely because this temporal window matches the characteristic correlation times of the breaking and re-forming dynamics of hydrogen bonds. On the contrary, slower motions of surface residues would be less affected by the interaction with the solvent.

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