Picosecond Internal Dynamics of Lysozyme as Affected by Thermal Unfolding in Nonaqueous Environment

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ABSTRACT   A neutron-scattering investigation of the internal picosecond dynamics of lysozyme solvated in glycerol as a function of temperature in the range 200–410 K has been undertaken. The inelastic contribution to the measured intensity is characterized by the presence of a bump generally known as "boson peak", clearly distinguishable at low temperature. When the temperature is increased the quasielastic component of the spectrum becomes more and more intrusive and progressively overwhelms the vibrational bump. This happens especially for $T > 345$ K when the protein goes through an unfolding process, which leads to the complete denaturation. The quasielastic term is the superposition of two components whose intensities and linewidths have been studied as a function of temperature. The slower component describes motions with characteristic times of ~4 ps corresponding to reorientations of polypeptide side chains. Both the intensity and linewidth of this kind of relaxations show two distinct regimes with a crossover in the temperature range where the melting process occurs, thus suggesting the presence of a dynamical transition correlated to the protein unfolding. Conversely the faster component might be ascribed to the local dynamics of hydrogen atoms caged by the nearest neighbors with characteristic time of ~0.3 ps.

INTRODUCTION

The physical characterization of native and denatured protein states is crucial to a complete understanding of protein thermal stability. This characterization requires a detailed investigation of the protein configurational distribution and internal dynamics before and after unfolding under various conditions, which can be varied for example by changing the parameters that may affect the protein thermal stability such as the solution pH (Burova et al., 2000) or the cosolvent mole fraction in aqueous solutions (Zaks and Klibanov, 1984). In fact, the protein thermal stability may be modulated also by properly choosing both the kind and the amount of molecules around the macromolecule surface. When proteins are solved or embedded in certain media termed "stabilizers", their thermal stability is sensibly enhanced and the so-called melting temperature increases remarkably. The prototypes of stabilizers are polyalcohols, such as sugars, glycerol, sugar-alcohols, and other more exotic substituted sugar molecules (Bell et al., 1995). Because of their properties, these substances are often used in an increasing number of applications, such as food processing, manufacture of pharmaceuticals and chiral molecules, storage of lyophilized biomolecules, long-term drug delivery, and environmental and clinical assay kits like biosensors (Tsai et al. 2000; Drago and Gibson, 2001).

Despite this large variety of applications, the knowledge at a molecular level of the mechanisms that determine the stabilizing nature of the medium around the protein is still incomplete. In fact, the protein flexibility (which can be regarded as an indicator of the ability in sampling its conformational rearrangements) is strongly affected by the environment that may forbid or allow jumps among different "conformational substates", i.e., nearly isoenergetic minima in the hypersurface of the protein potential energy (Frauenfelder et al., 1991). It is commonly accepted that stabilizer substances lower the protein flexibility through more or less severe constraints on conformational and dynamical degrees of freedom of these biomolecules (Gottfried et al., 1996; Shamblin et al., 1999; Lichtenegger et al., 1999; Cordone et al., 1999). The opposite action is performed by plasticizer media, which confer to proteins an improved ability in rearranging among different configurations (Gregory, 1995). It should be remarked that, when water, which is the archetype of plasticizer milieus, is added to protein-stabilizer solutions or mixtures, their thermal stability is sensibly deteriorated and this effect is more and more evident as the hydration level increases (Bell et al., 1995). With this respect, thermal stability has been recently supposed to be inversely correlated to protein flexibility (Tang and Dill, 1998; Tsai et al., 2001) even if this point is still largely debated (Fitter et al., 2001; Tehei et al., 2001). It is worth noting that an essential contribution to protein flexibility is provided by motions on the picosecond time-scale, which are fast stochastic thermal fluctuations involving structural relaxations such as the reorientation of protein side chains (Fitter et al., 1996). Molecular dynamics simulations and neutron-scattering experiments have demonstrated that this kind of dynamical contribution, which seems to be partly responsible for the activation of biological functionality of proteins (Seno and Go, 1990; Ferrand et al., 1993; Marques and Sanjouand, 1995; Vitkup et al., 2000), shows an anomalous trend as a function of the temperature. The atomic mean square displacements of hydrated protein powders, that describe on the average the extent of picosecond structural relaxations, exhibit a well-defined dynamical transition, i.e., a marked anharmonic departure at a certain temperature T_d from the low-T harmonic behavior

(Doster et al., 1989). Very recently we have found that when lysozyme is solvated in a glycerol-water mixture, both the extent of its mean square displacements above T_d and T_d itself are strongly affected by the hydration degree (Paciaroni et al., 2002). This result shows that the dynamical transition features, and more generally the molecular dynamics on the picosecond timescale, may be finely tuned by changing the environment around the protein (Frauenfelder and McMahon, 1998). With this respect it is important to characterize the dynamics of both the native and the denatured state of proteins when they are solvated in glass forming solvents such as glycerol, to better understand how the dynamics itself is related to the improved thermal stability. We have then performed a detailed neutron-scattering investigation to study the picosecond internal dynamics of lysozyme solvated with glycerol where the protein is in its native state (Bell et al., 1995). The dynamical evolution of lysozyme in this nonaqueous environment has been studied in a wide temperature range, from the low-temperature harmonic condition up to the high-temperature denatured state. Such system has been recently studied on the same temperature range (Tsai et al., 2000, 2001). In this article we show that it is possible to accurately single out the purely quasielastic contribution to the spectra by properly subtracting a reasonable approximation of the inelastic part from the total signal. Thanks to this data analysis procedure and to the very good statistics we collected, we found that a two-component model describes in an excellent way the quasielastic signal, i.e., the relaxational lysozyme internal dynamics on the picosecond timescale. These two distinct contributions correspond to relaxational distributions with relevant characteristic times of $\sim$4 ps and $\sim$0.3 ps, representing, respectively, the collective and local dynamics of a variety of protein molecular subunits, such as methyl and larger polypeptide side chains. The two-component description is valid on the whole temperature range, also above the melting temperature. On these grounds we were able to reveal for the first time the clear presence of a dynamical transition related to the melting process involving the slower quasielastic component, which shows a crossover in both the intensity and the linewidth just in coincidence with the onset of protein unfolding. Such behavior suggests that this kind of motion is able to trigger the structural and dynamical mechanisms associated to protein thermal denaturation.

MATERIAL AND METHODS

Sample preparation

Hen egg white lysozyme has been purchased from Sigma (St. Louis, MO) and used without further purification. To emphasize the incoherent signal from nonexchangeable protein hydrogen atoms the sample preparation has been performed by using only fully deuterated glycerol and heavy water (Sigma). An amount of lysozyme of 2 g was dissolved in 20 g of D_2O at room temperature and left for seven days to properly substitute exchangeable hydrogen atoms with deuterium. On the basis of what was reported by Gregory and Lumry (1985), we estimated that very slow-exchangeable

hydrogens, possibly released only during the unfolding process, would provide a spurious contribution to the incoherent quasielastic scattering due to the proton exchanging with the solvent by <0.5% of the total signal. This is in agreement with prompt γ activation results on the same system (Tsai et al., 2001), which indicate that the amount of nonexchanged hydrogens is not appreciable. Thus the contribution of residual exchangeable hydrogens to the scattering can be considered negligible in very good approximation. The solution was freeze dried into a powder, which was then desiccated under vacuum in the presence of P_2O_5 to achieve a water content as low as possible. The protein was then dissolved in a solution of D_2O + deuterated glycerol and lyophilized again to obtain a mixture of dry lysozyme and glycerol in the weight proportion of 1:1.

Neutron-scattering spectroscopy

In a neutron-scattering experiment the relevant and measured quantity is the dynamical structure factor $S(q,\omega)$, which provides the probability for an incident neutron to be scattered by the sample with a wavevector transfer $\hbar \mathbf{q}$ and an energy transfer $\hbar \omega$, where $\hbar$ is the reduced Planck constant (Lovesey, 1988; Bée, 1988). The ω and q-dependence of $S(q,\omega)$ carries information on the characteristic correlation times and on the spatial geometry of the observed molecular motions, respectively. Because the sample is isotropic to a very good approximation, the dynamical structure factor depends effectively on the modulus q of the wavevector transfer rather than on the vector $\mathbf{q}$.

The dynamical structure factor contains both coherent and incoherent scattering contributions, arising from interparticle and self-correlation, respectively. Hydrogen atoms have an incoherent scattering cross section (80.27 b) more than one order of magnitude larger than the total scattering cross section of most elements and isotopes. Particularly the difference between the incoherent scattering cross section for hydrogen and deuterium (2.05 b) atoms is huge (Lovesey, 1988).

Because hydrogen atoms are abundantly and quasiuniformly distributed throughout the whole lysozyme protein, a complete sampling of all the molecular motions within the observed dynamical window is guaranteed. Because the lysozyme had been solvated with deuterated glycerol, the signal we detected is mainly due to nonexchangeable lysozyme hydrogen atoms and can be reasonably considered as incoherent to a very good approximation.

In the incoherent approximation, the dynamical structure factor is generally expressed as (Orecchini et al., 2002; Paciaroni et al., 2003):

$$S(q, \omega)$$
$$= e^{-\langle u^2 \rangle q^2} \left[A_0(q)\delta(\omega) + \sum_{i=1}^{n} A_i(q) S_{qel,i}(q, \omega) + S_{inel}(q, \omega) \right]$$
$$\otimes R(q, \omega). \tag{1}$$

In Eq. 1 the dynamical structure factor is split into the sum of three components. The first term $A_0(q)\delta(\omega)$ represents the elastic response of the system, whose energy dependence is accounted for by a Dirac delta function. The q-dependence is provided by the elastic incoherent scattering factor $A_0(q)$, which in fact represents the space-Fourier transform of the scatterers' distribution, taken at infinite time and averaged over all the possible initial positions. The second term is the quasielastic scattering contribution, which manifests itself in the experimental spectra as a broadening of the elastic peak and accounts for n different possible kinds of relaxation motions, sampled by the hydrogen atoms. We describe each of these relaxations as the product of the quasielastic incoherent structure factor (QISF) $A_i(q)$ and a properly normalized function $S_{qel,i}(q,\omega)$.

In fact, due to the large variety of quasielastic components in protein spectra, every function can be regarded as a broad, almost continuous distribution of motions rather than a single kind of movement. Each one of these distributions is characterized by its own linewidth Γ_i (half width at half maximum (HWHM)), which is related to motion with characteristic time

$\tau_i = \hbar/\Gamma_i$. For each temperature the elastic and quasielastic structure factors are related through the sum rule $\sum_{i=0}^{n} A_i(q) = 1$.

The third term is the inelastic incoherent scattering function that is proportional to $g(\omega)n(\omega, T)/\omega$ where $g(\omega)$ is the protein density of vibrational states and $n(\omega, T) = (e^{\hbar\omega/k_BT} - 1)^{-1}$ is the Bose factor (Lovesey, 1988; Bée, 1988). Finally due to the finite resolution of the spectrometer, Eq. 1 must be convoluted with the experimental resolution function $R(q, \omega)$. At the elastic peak energy this function is provided by the vanadium standard measurement. The so-called Debye-Waller factor in front of Eq. 1 describes the Gaussian q decreasing due to the vibrational atomic mean square displacements $\langle u^2 \rangle$. The temperature dependence of the $\langle u^2 \rangle$'s has been estimated through the Einstein model for solids by using the fitting parameters obtained in a previous investigation (Paciaroni et al., 2002).

Neutron-scattering experiment

The measurements were performed on the high-flux time-of-flight spectrometer IN6, at ILL (Institut Laue-Langevin, Grenoble). An incident wavelength $\lambda = 5.1$ Å was employed, achieving a q-range from 0.2 Å^{-1} to 2.0 Å^{-1}, an accessible energy transfer range from -2 meV to 2 k_BT and an average energy resolution with a full width of $\sim$90 μeV. This energy range and resolution allow to probe motions with characteristic times faster than $\sim$15 ps.

The standard slab-shaped aluminum cell with a thickness of 0.5 mm, has been placed into the neutron flux with an angle of 135° with respect to the incident beam. In the temperature range 330–370 K the protein goes through the unfolding process with temperature-dependent kinetics. As we are mainly concerned with the characterization of protein native versus denatured state, the neutron-scattering spectra were collected only in the ranges 200–330 K and 370–410 K where the protein is in a definite and stable native or denatured state, respectively, as it turns out from differential scanning calorimetry measurements (see *inset*, Fig. 5). Before any data processing the raw data were corrected for empty cell contribution, self-shielding and self-absorption and normalized to a vanadium standard to take into account the not uniform detector efficiency as a function of the scattering angle. All the spectra shown in this article have been obtained averaging over the whole angular range. This corresponds to an average scattering angle $2\theta_{av}$ of 66° and an average q at the elastic peak of 1.3 Å^{-1}. Due to the high value of the transmission coefficient ($t(90°) = 0.91$) no correction for multiple scattering has been applied.

The inelastic and quasielastic neutron scattering

To obtain as precisely as possible the quasielastic structure factor as a function of temperature, the vibrational contributions to the total dynamical structure factor have to be carefully evaluated. To this purpose an already applied strategy (Cusack, 1989; Cusack and Doster, 1990) is to measure the scattering spectrum for the sample at low temperature ($T = 200$ K). This strategy is based on the hypothesis that harmonic and anharmonic motions are statistically independent. At low temperature, where the negligible presence of quasielastic scattering has been checked, the dynamical structure factor is just the sum of the elastic and inelastic terms:

$$S(q, \omega) = e^{-\langle u^2 \rangle q^2} [A_0(q)\delta(\omega) + S_{inel}(q, \omega)] \otimes R(q, \omega). \quad (2)$$

The elastic term has been described taking account of the standard measurement on a vanadium sample. The inelastic contribution at low temperature can be described in an excellent way by an analytic empirical function. In the harmonic approximation the inelastic structure factor depends on temperature only through the Debye-Waller factor and the Bose factor and we can calculate the inelastic contribution at any temperature T by rescaling the inelastic term at a reference temperature of $T_0 = 200$ K simply through the relationship (Diehl et al., 1997; Paciaroni et al., 1999):

$$S_{inel}^T(q, \omega) = S_{inel}^{T_0}(q, \omega) \frac{e^{-\langle u^2(T) \rangle q^2}}{e^{-\langle u^2(T_0) \rangle q^2}} \frac{n(\omega, T)}{n(\omega, T_0)}. \quad (3)$$

Finally a careful subtraction of the vibrational part from the total spectrum provides a reliable estimation of the superposition of the elastic and quasielastic terms (Cusack, 1989; Cusack and Doster, 1990) at a temperature T:

$$\Delta S^T(q, \omega) = [S^T(q, \omega) - S_{inel}^T(q, \omega)] \otimes R(q, \omega), \quad (4)$$

and more explicitly:

$$\Delta S^T(q, \omega) = e^{-\langle u^2 \rangle q^2} \left[A_0(q)\delta(\omega) + \sum_{i=1}^{n} A_i(q)S_{qel,i}(q, \omega) \right]$$
$$\otimes R(q, \omega). \quad (5)$$

RESULTS AND DISCUSSION

In Fig. 1 the measured dynamical structure factor from lysozyme hydrogen atoms is shown at four selected temperatures. At this stage the spectrum can be considered as the sum of three terms, i.e., the elastic, quasielastic, and inelastic contributions as we mentioned in the Material and Methods section (see above). In fact the elastic peak is certainly not a $\delta(\omega)$ signal because of the finite experimental energy resolution. The linewidth of this resolution function provides the lower limit for observable motions in the system. The elastic intensity diminishes with temperature according to the Debye-Waller factor in Eq. 1, due to the increasing atomic mobility (Doster et al., 1989; Andreani et al., 1995; Paciaroni et al., 1999). The decrease by >50% over all the explored temperature range of the elastic intensity is compensated by the strong rising of the quasielastic and inelastic signal. The spectra show two main features: the intense elastic peak and an excess of scattering centered at $\sim$4 meV, particularly evident at 200 K. It is evident that if the low-temperature spectrum is superimposed to the vanadium spectrum, at this temperature the quasielastic contribution is <2–3% with respect to the 300-K spectrum. In fact as soon as the
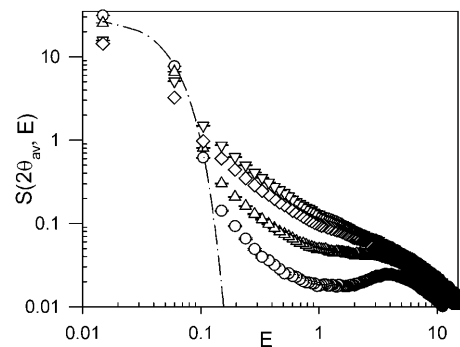

FIGURE 1 Experimental incoherent structure factor of lysozyme in deuterated glycerol. ($\bigcirc$) $T = 200$ K; ($\triangle$) $T = 300$ K; ($\diamondsuit$) $T = 370$ K; ($\triangledown$) $T = 400$ K. The dash-dot line is the resolution function as derived from the vanadium standard measurement.

temperature increases this peak becomes less and less distinguishable due to the overwhelming presence of the quasielastic scattering. At 300 K the vibrational bump already looks like a shoulder and is no longer perfectly resolved, whereas at 370 K and above the quasielastic signal is "overflowing" the inelastic region.

In Fig. 2 we focused our attention on the quasielastic and inelastic structure factor for the spectra at 200 K and at 300 K, 330 K, and 400 K rescaled by the Bose factor. For $\hbar\omega > 5$ meV, the intensities are perfectly superimposed, thus suggesting that in this energy range the scattering is purely inelastic, i.e., the quasielastic contribution becomes negligible. In addition such a good matching in the inelastic regions of the spectra testifies the goodness and reliability of the rescaling procedure. Conversely for $\hbar\omega < 5$ meV there is not superimposition between the low-temperature spectrum and the rescaled spectra at higher temperatures because of the onset of quasielastic scattering. This quasielastic contribution comes from the activation of diffusive confined and anharmonic protein motions due to the so-called dynamical transition above ~235 K (Paciaroni et al., 2002) already emphasized with a break in the atomic mean square displacements versus T behavior (Doster et al., 1989; Paciaroni et al., 2002a). The rising quasielastic intensity deforms the shape of the inelastic low-energy bump resulting in an apparent shift of the peak maximum to lower energy. This vibrational peak, which is well resolved at low temperature, is reminiscent of the "boson peak" already observed in other biological systems (Fitter, 1999; Paciaroni et al., 1999, 2003) and glassy materials (Elliot, 1992; Frick and Richter, 1995). The features, the behavior, and the possible origin of this low-frequency anomaly, which represents an excess of scattering over the Debye level, has been largely discussed and debated in the past (Elliot, 1992; Frick and Richter, 1995; Paciaroni et al., 1999; Fitter, 1999) and also very recently (Grigera et al., 2003). In hydrated protein powders, this bump is clearly visible at low temperature but is rather

overwhelmed by the quasielastic scattering as soon as the temperature reaches 300 K (Doster et al., 1990; Ferrand et al., 1993; Paciaroni et al., 1999; Orecchini et al., 2002). On the contrary, when the protein is dissolved in glycerol, the inelastic excess is still recognizable even at 300 K and above, although it is progressively merging with the rising quasielastic contribution, which is less pronounced than in hydrated protein powders as very recently shown by Raman scattering measurements (Caliskan et al., 2003). This fact seems to suggest a role of glycerol in conditioning the vibrational properties of the protein. The interaction with the glycerol molecules preserves to some extent the vibrational stiffness of the macromolecule. This idea is supported by a slight shift of the boson peak of lysozyme in glycerol toward higher energy with respect to the position of the peak for hydrated protein powder (~3 meV) (Cusack and Doster, 1990; Orecchini et al., 2002). If we very simply schematize the boson peak as a distribution of harmonic oscillators, this shift to higher energy would correspond to force constants bigger in lysozyme when glycerol rather than water molecules surround its surface. Therefore both the persistence at higher temperatures of the low-energy vibrational properties and the decreased amount of quasielastic scattering observed in this nonaqueous environment indicates that glycerol reduces the protein flexibility. This result is in agreement with a recent elastic neutron-scattering investigation showing that the atomic mean square displacements in lysozyme solvated in glycerol are lower than in hydrated powder samples (Tsai et al., 2000).

The protein dynamics is largely affected by the environment, which is seen by the surface moiety of the macromolecule. The specific interaction between the surface and the molecules of stabilizer nonaqueous organic solvents shifts the onset of anharmonic motions to higher temperature and reduces also their extent to a greater degree compared to what happens in water. As we mentioned in the Introduction section it is even possible, in principle, to modulate and fine tune such dynamics by changing in a suitable way the composition of the external environment, for example, slowly increasing the percentage of water in the mixture (Paciaroni et al., 1999, 2002; A. Paciaroni, S. Cinelli, A. De Francesco, and G. Onori, unpublished data).

To properly describe the quasielastic contribution, the inelastic term has been subtracted to the measured spectra at all the temperatures through Eq. 4. In Fig. 3 the difference spectra at four selected temperatures are shown. The continuous increasing of the quasielastic scattering as a function of T and the consequent progressive activation of the protein internal relaxation dynamics is definitely evident.

It should be observed that most of the quasielastic signal originates from confined diffusive side-chain reorientation as most of the nonexchangeable hydrogens of lysozyme belong just to side groups (~82%). The large number of different polypeptide side groups and their possible individual motions
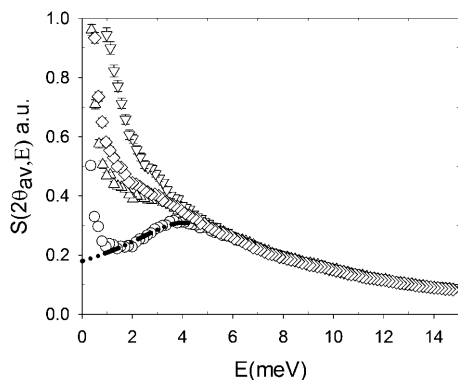
FIGURE 2 Experimental incoherent structure factor of lysozyme in deuterated glycerol obtained after rescaling by the Bose factor to a common temperature $T_0 = 200$ K. (○) $T = 200$ K; (△) $T = 300$; (◇) $T = 330$ K; (▽) $T = 400$ K. The dash-dot line is the best fit for the inelastic scattering at 200 K.
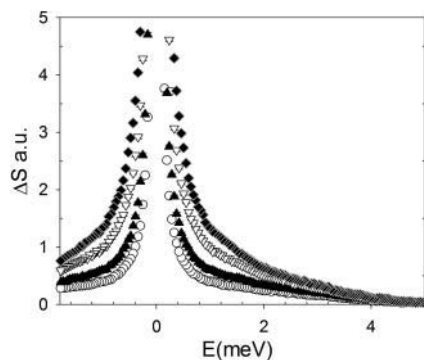
FIGURE 3 Quasielastic neutron-scattering spectra of lysozyme in deuterated glycerol for different temperatures: from the bottom to the top $T = 300$ K, $T = 330$ K, $T = 370$ K, $T = 400$ K. The inelastic contribution has been subtracted according to the procedure described in the text (see Eq. 4).

FIGURE 4 Quasielastic neutron-scattering spectrum of lysozyme embedded in deuterated glycerol at $T = 300$ K (*a*) and $T = 400$ K (*b*). The dot and the dash-dot lines represent the Gaussian and the Lorentzian components, respectively. The solid line is the best fit to the experimental data in the range -2–5 meV.

are responsible for a huge variety of stochastic protein thermal fluctuations occurring in the window timescale 10^{-1}–10^2 ps (Fitter et al., 1996). To describe quantitatively this behavior we have applied Eq. 5 where, as we mentioned above, the terms $S_{qel,i}$ have to be considered as continuous distributions of motions rather than single kinds of movements.

The most traditional approach (Receveur et al., 1997; Fitter et al., 1997, 1999; Paciaroni et al., 1999; Orecchini et al., 2002; Russo et al., 2002) to fit the quasielastic term in biological systems consists in using a sum of Lorentzian functions (generally two or three components). A different approach to model the quasielastic structure factor has been undertaken by Tsai et al. (2001) just in analyzing lysozyme solvated with glycerol. They fitted the spectra by the Fourier transform of the Kohlrausch-Williams-Watts function (Williams and Watts, 1970). However a direct comparison of their results with ours is not really feasible as they collected data with a lower energy resolution, and no inelastic scattering contribution has been subtracted. The spectra we analyzed seem to display a different shape both near the elastic peak and on the meV energy domain. In fact the cited approaches do not fit the already-reduced spectra, where the inelastic contribution has been taken off considering the above approximation. We have found that our data are fitted in an excellent way by a combination of two terms Lorentzian and Gaussian shaped, which described the narrow and the wide quasielastic contributions, respectively. In Fig. 4 the quasielastic spectra at 300 K (*a*) and 400 K (*b*) are plotted together with the fit curves. The dramatic rising of the quasielastic intensity due to the increasing of temperature can be noted immediately. The choice of a Gaussian distribution rather than a Lorentzian one to fit the data above ~ 1 meV is suggested by the faster falling down of the high energy tails of the spectra.

In Fig. 5 the QISFs of the two quasielastic contributions are shown. The Gaussian curve seems to be representative of a fast local dynamics arising at around 240 K, as its intensity vanishes approximately at that temperature when one
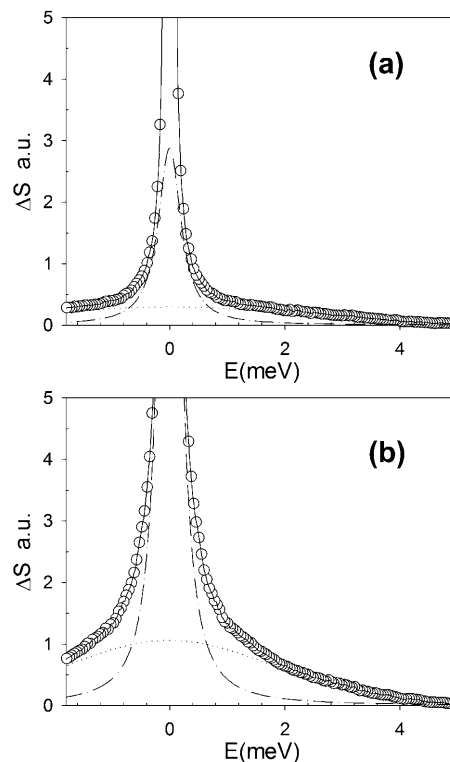
extrapolates linearly the corresponding QISF. This suggests that this component can be related to the onset of anharmonic motions appearing just above the dynamical transition of lysozyme solvated in glycerol at $T_G \sim 235$ K (Paciaroni et al., 2002). Such kinds of fast relaxations are characterized by a typical average time of ~ 0.3 ps that seems to be independent of the environment, as the same value has been found also in protein hydrated powders (Doster et al., 1989; Fitter et al., 1997, 1999), even if their amplitudes are sensibly suppressed by glycerol (Caliskan et al., 2003). The linear temperature dependence of the corresponding QISF suggests that this Gaussian component is not affected as the protein goes through the unfolding process and the molecular surroundings of each moving protein subunit change dramatically. The temperature only seems to pilot the amount of this dynamics by controlling the number of protein groups that participate in the motions and their amplitudes.

According to a mode-coupling-theory-based (Göetze, 1991) description of the protein dynamical behavior (Doster et al., 1989, 1990), such a fast term is reminiscent of the so-called β-process that can be attributed to local motions of particles caged in a heat bath of nearest neighbors. The HWHM of this fast vibrational-like component, shows a very slight decreasing dependence on temperature assuming values in the range 1.8–2.3 meV suggesting a light soften-
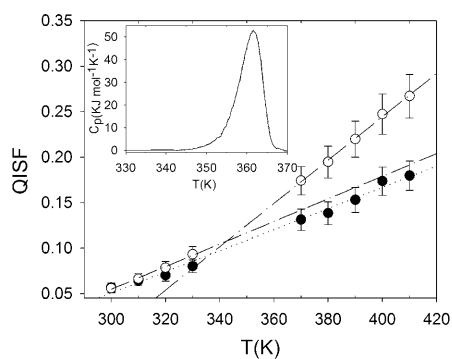
FIGURE 5 Quasielastic incoherent neutron-scattering factors: slow-Lorentzian (○) and fast-Gaussian (●) component; dash-dot and dotted lines are linear fits of the data. (*Inset*) Denaturation thermogram of lysozyme in glycerol measured by differential scanning calorimetry.

FIGURE 6 HWHM of the slow quasielastic Lorentzian component as a function of temperature.

ing of the observed system. As the temperature increases, the cage formed by the neighbor atoms of the vibrating hydrogens becomes more and more loose and so become the recall constants of the interaction forces, which guarantee the three-dimensional protein structure.

The QISF of the Lorentzian curve is also an increasing function of temperature presenting, however, a remarkable break at ~345 K just in coincidence with the beginning of the melting process, as it is shown on the thermogram of the Fig. 5 inset. It should be remarked that the present differential scanning calorimetric measurements were performed on the same samples investigated through neutron-scattering experiments, with deuterated glycerol as well, to exclude any isotopic effect. The melting temperature is ~362 K, sensibly higher than that of highly hydrated lysozyme powders, $T_m \approx 340$ K (Fujita and Noda, 1978), due to the stabilizer character of glycerol. This component is representative of a collection of slower diffusive collective motions with characteristic times of ~4 ps. An average characteristic time of the same order of magnitude has been recently found in α-amylase from *Bacillus licheniformis* in aqueous environment, where it was attributed to the reorientation of protein side chains (Fitter, 2003). According to the "diffusion inside a sphere" model (Volino and Dianoux, 1980), these motions are confined within a spherical volume, whose radius (of the order of the Å) increases as the protein unfolds or the temperature goes up (Fitter, 2003). Actually, the increasing trend of the QISF shown in Fig. 6 could depend on the number of effective scattering subunits participating to the diffusive motions or on the enlargement of the volume explored by the moving subunits and therefore on a bigger amplitude of the relevant motion itself. By a preliminary analysis of the QISF as a function of q and T (A. Paciaroni, A. De Francesco, and G. Onori, unpublished data) it seems that in our case the quasielastic scattering intensities grow with temperature mostly because larger amplitudes of the diffusive motions are attained, in agreement with the mentioned results by Fitter (2003). The break
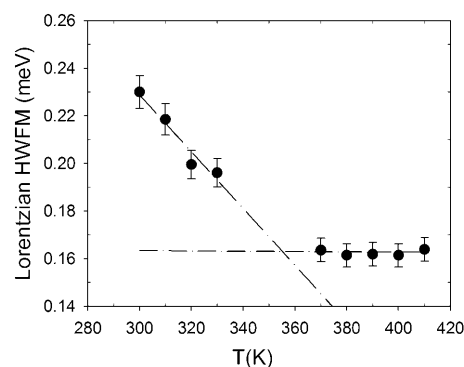
at $T = 345$ K (Fig. 5) marks a crossover between two different regimes as far as concern the temperature response of the system, being the unfolded state characterized by a higher T-sensitivity with respect to the native state. This effect seems to be related to the larger volume accessible to side groups once the protein denaturates.

The trend of the Gaussian and Lorentzian QISF shows that, before the melting process transition temperature, the 0.3 ps and the 4 ps components respond identically to the rising temperature, suggesting some correlation between the two kind of relaxations. Conversely they become independent one from the other when the transition starts.

Looking at the linewidth of the Lorentzian quasielastic component an unexpected behavior occurs. Initially the distribution linewidth decreases with temperature and after the melting transition starting temperature it reaches a constant value (Fig. 6). Because of the inverse relation between linewidth and correlation time of the associated relaxation motion this would suggest an apparent slowing down of the protein dynamics in the range 300–330 K. This narrowing would correspond to a change of the distribution shape, due to the appearance of slow motions related to more massive protein subunits as the temperature increases.

To better understand the specific role of the environment, it would be interesting to compare the dynamical behavior of lysozyme solvated in glycerol with that of lysozyme in aqueous solvent. At the best of our knowledge, the only study of the lysozyme internal dynamics in aqueous environment in the picosecond timescale in the folded and unfolded state has been done by Tsai et al. (2001) but, as already said, their results cannot be directly compared with ours. It is worth mentioning that, before denaturation, in hydrated protein powders the slow component distribution linewidth broadens with temperature (Doster et al., 1989). This fact suggests that the slow picosecond dynamics behaves differently when the surface protein moiety interacts with glycerol rather than with water molecules. Due to its high viscosity the glycerol environment damps and hinders low-frequency more than high-frequency diffusive motions. This key role of the solvent viscosity in conditioning the

picoseconds protein dynamics has been recently stressed by a molecular dynamics simulations study (Walser and van Gunsteren, 2001). On a microscopic point of view the massive subunits need a greater effort to relax because of their larger size that could hinder the reorientation processes. As soon as the temperature can guarantee favorable viscosity conditions, glycerol molecules are able to support such low-frequency motions, which begin to explore larger volumes and attain bigger amplitudes. On the other hand we could speculate that when the protein is in the presence of an aqueous environment, both low- and high-frequency relaxations are less sensibly affected by the viscosity change versus T.

After the melting transition the linewidth constant value indicates that the distribution shape does not change any more. In fact in this temperature range the subunits relevant for this slow dynamics experience similar environment conditions and are able to relax rather independently from the temperature itself and hindering effects. This picture is in agreement with the finding of Tsai et al. (2001) showing how after the melting transition the conformational landscape remains constant even at higher increasing temperature. The breaks visible in both the Lorentzian QISF and relative linewidth are the signature of a dynamical transition that determines the onset of diffusive motions possibly involved in priming the denaturation process.

Regarding the quasielastic scattering intensity as a measure of the protein internal dynamics, it can be concluded that its flexibility increases with temperature. In particular not only the flexibility of the unfolded state of lysozyme in glycerol is higher than that of the native state but it is more sensitive to temperature variation and is mainly affected by the growing amplitudes of the slow diffusive motions. The finding that the protein unfolding is related to a loss of rigidity is in agreement with other neutron-scattering experimental studies on proteins in solution (Receveur et al., 1997; Russo et al., 2002; Fitter, 2003) and theoretical results (Rader et al., 2002).

CONCLUSIONS

We performed a neutron-scattering investigation on lysozyme solvated in glycerol as function of temperature, by monitoring the protein dynamics with a special attention to the changes occurring in correspondence with the unfolding process. Once the purely vibrational contribution had been evaluated from the low-temperature spectrum, we could verify that the extent of the diffusive dynamics of polypeptide side chains is somehow restricted by the glycerol environment with respect to water. This is witnessed by the presence of the vibrational feature also known as boson peak until and above room temperature. The quasielastic scattering is properly described as the superposition of two components. The first one is a wide Gaussian function, which represents a collection of fast ($\tau = 0.3$ ps) local motions. The other component is a narrow Lorentzian function, which describes a distribution of slower ($\tau = 4$ ps) collective relaxations. Because two distinct regimes with a crossover in the protein melting range are well distinguishable for this component, in the trend of both QISF and linewidth with the temperature, it was argued that such kind of dynamics is markedly involved in the unfolding process. In particular, the linewidth behavior suggests that within the distribution of motions described by this component, the large amplitude slow motions are progressively permitted as the temperature increases basically due to the lowering of glycerol viscosity.

REFERENCES

Andreani, C., A. Filabozzi, F. Menzinger, A. Desideri, A. Deriu, and D. Di Cola. 1995. Dynamics of hydrogen atoms in superoxide dismutase by quasielastic neutron scattering. *Biophys. J.* 68:2519–2523.

Bée, M. 1988. Quasielastic Neutron Scattering. Adam Hilger, Bristol, UK and Philadelphia, PA.

Bell, L. N., M. J. Hageman, and L. M. Muraoka. 1995. Thermally induced denaturation of lyophilized bovine somatotropin and lysozyme as impacted by moisture and excipients. *J. Pharmacol. Sci.* 84:707–712.

Burova, T. V., N. V. Grinberg, V. Y. Grinberg, R. V. Rariy, and A. M. Klibanov. 2000. Calorimetric evidence for a native-like conformation of hen egg-white lysozyme dissolved in glycerol. *Biochim. Biophys. Acta.* 1478:309–317.

Caliskan, G., A. Kisliuk, A. M. Tsai, C. L. Soles, and A. P. Sokolov. 2003. Protein dynamics in viscous solvents. *J. Chem. Phys.* 118:4230–4236.

Cordone, L., M. Ferrand, E. Vitrano, and G. Zaccai. 1999. Harmonic behaviour of trehalose-coated carbon-monoxy-myoglobin at high temperature. *Biophys. J.* 76:1043–1047.

Cusack, S. 1989. Low frequency dynamics of proteins: comparison of inelastic neutron scattering results with theory. *Chem. Scr.* 29A:103–107.

Cusack, S., and W. Doster. 1990. Temperature dependence of the low frequency dynamics of myoglobin. *Biophys. J.* 58:243–251.

Diehl, M., W. Doster, W. Petry, and H. Shober. 1997. Water-coupled low frequency modes of myoglobin and lysozyme observed by inelastic neutron scattering. *Biophys. J.* 73:2726–2732.

Doster, W., S. Cusack, and W. Petry. 1989. Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 337:754–756.

Doster, W., S. Cusack, and W. Petry. 1990. Dynamics instability of liquid like motions in a globular protein observed by inelastic neutron scattering. *Phys. Rev. Lett.* 65:1080–1083.

Drago, G. A., and T. D. Gibson. 2001. Enzyme stability and stabilisation: applications and case studies. *In* Engineering and Manufacturing for Biotechnology. M. Hofman and P. Thonart, editors. Kluwer Academic Publishers, Dordrecht, Holland. 361–376.

Elliot, S. R. 1992. A unified model for the low-energy vibrational behaviour of amourphous solids. *Eurphys. Lett.* 19:201–206.

Ferrand, M., A. Dianoux, and W. Petry. 1993. Thermal motion and function of bacteriorodhopsin. *Proc. Natl. Acad. Sci. USA.* 90:9668–9672.

Fitter, J. 2003. A measure of conformational entropy change during thermal protein unfolding using neutron spectroscopy. *Biophys. J.* 84:3924–3930.

Fitter, J. 1999. The temperature dependence of internal molecular motions in hydrated and dry α-amylase: the role of hydration water in the dynamical transition of proteins. *Biophys. J.* 76:1034–1042.

Fitter, J., R. Herrmann, N. A. Dencher, A. Blume, and T. Hauss. 2001. Activity and stability of a thermostable α-amilase compared to its mesophilic homologue: mechanism of thermal adaptation. *Biochemistry.* 40:10723–10731.

Fitter, J., R. E. Lechner, and N. A. Dencher. 1997. Picosecond molecular motions in bacteriorhodopsin from neutron scattering. *Biophys. J.* 73: 2126–2137.

Fitter, J., R. E. Lechner, G. Buldt, and N. A. Dencher. 1996. Internal molecular motions of bacteriorhodopsin: hydration-induced flexibility studied by quasielastic inchoerent neutron scattering using oriented purple membranes. *Proc. Natl. Acad. Sci. USA.* 93:7600–7605.

Frauenfelder, H., and B. McMahon. 1998. Dynamics and function of proteins: the search for general concepts. *Proc. Natl. Acad. Sci. USA.* 9995:4795–4797.

Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science.* 254:1598–1603.

Frick, B., and D. Richter. 1995. The microscopic basis of the glass transition in polymers from neutron scattering studies. *Science.* 267: 1939–1945.

Fujita, Y., and Y. Noda. 1978. Effect of hydration on the thermal denaturation of lysozyme as measured by differential scanning calorimetry. *Bull. Chem. Soc. Jpn.* 51:1567–1568.

Göetze, W. 1991. Liquids, Freezing and the Glass Transition. North Holland, Amsterdam, The Netherlands.

Gottfried, D. S., E. S. Peterson, A. G. Sheikh, J. Wang, and J. M. Friedman. 1996. Evidence for damped hemoglobin dynamics in a room temperature trehalose glass. *J. Phys. Chem.* 100:12034–12042.

Gregory, R. B. 1995. Protein-Solvent Interactions. Marcel Dekker, New York.

Gregory, R. B., and R. Lumry. 1985. Hydrogen-exchange evidence for distinct structural classes in globular proteins. *Biopolymers.* 24:301–326.

Grigera, T. S., V. Martin-Major, G. Parisi, and P. Verrocchio. 2003. Phonon interpretation of the boson peak in supercooled liquids. *Lett. to Nature.* 422:289–292.

Lichtenegger, H., W. Doster, T. Kleinert, A. Birk, F. Parak, B. Sepiol, and G. Vogl. 1999. Heme-solvent coupling, a Mössbauer study of myoglobin in sucrose. *Biophys. J.* 76:414–422.

Lovesey, S. 1988. Theory of Neutron Scattering from Condensed Matter. Oxford University Press, Oxford, UK.

Marques, O., and Y. H. Sanjouand. 1995. Hinge-bending motion in citrate synthase arising from normal modes calculations. *Proteins.* 23:557–560.

Orecchini, A., A. Paciaroni, A. R. Bizzarri, and S. Cannistraro. 2002. Dynamics of different hydrogen classes in β-lactoglobulin: a quasielastic neutron scattering investigation. *J. Phys. Chem. B.* 106:7348–7354.

Paciaroni, A., S. Cinelli, and G. Onori. 2002. Effect of the environment on the protein dynamical transition: a neutron scattering study. *Biophys. J.* 83:1157–1164.

Paciaroni, A., A. Orecchini, S. Cinelli, G. Onori, R. E. Lechner, and J. Pieper. 2003. Proteins dynamics on the picoseconds timescale as affected by the environment: a quasielastic neutron scattering study. *Chem. Phys.* 292:397–404.

Paciaroni, A., M. E. Stroppolo, C. Arcangeli, A. R. Bizzarri, A. Desideri, and S. Cannistraro. 1999. Incoherent neutron scattering of copper azurin: a comparison with molecular dynamics simulation results. *Eur. Biophys. J.* 28:447–456.

Rader, A. J., B. M. Hespenheide, L. A. Kuhn, and M. F. Thorpe. 2002. Protein unfolding: rigidity lost. *Proc. Natl. Acad. Sci. USA.* 99:3540–3545.

Receveur, V., P. Calmettes, J. C. Smith, M. Desmadril, G. Coddens, and D. Durand. 1997. Picosecond dynamical changes on denaturation of yeast phosphoglycerate kinase revealed by quasielastic neutron scattering. *Proteins.* 28:380–387.

Russo, D., J. Péz, J. M. Zanotti, M. Desmadril, and D. Durand. 2002. Dynamic transition associated with the thermal dentauration of small beta protein. *Biophys. J.* 83:2792–2800.

Seno, Y., and N. Go. 1990. Deoxymyoglobin studied by the conformational normal mode analysis. 2. The conformational change upon oxygenation. *J. Mol. Biol.* 216:111–126.

Shamblin, S., X. Tang, L. Chang, B. Hancock, and M. Pikal. 1999. Characterization of the time scale of molecular motion in pharmaceutically important glasses. *J. Phys. Chem. B.* 103:4113–4121.

Tang, K. E. S., and K. Dill. 1998. Native protein fluctuations: the conformational-motion temperature and the inverse correlation of protein flexibility with protein stability. *J. Biomol. Struct. Dyn.* 16:397–411.

Tehei, M., D. Madern, C. Pfister, and G. Zaccai. 2001. Fast dynamics of halophilicmalate dehydrogenase and BSA as measured by neutron scattering under various solvent conditions influencing protein stability. *Proc. Natl. Acd. Sci. USA.* 98:14356–14361.

Tsai, A. M., D. A. Neumann, and L. N. Bell. 2000. Molecular dynamics of solid-state lysoyme as affected by glycerol and water: a neutron scattering study. *Biophys. J.* 79:2728–2732.

Tsai, A. M., T. J. Udovic, and D. A. Neumann. 2001. The inverse relationship between protein dynamics and thermal stability. *Biophys. J.* 81:2339–2343.

Vitkup, D., D. Ringe, G. A. Petsko, and M. Karplus. 2000. Solvent mobility and the protein ''glass'' transition. *Nat. Struct. Biol.* 7:34–38.

Volino, F., and A. J. Dianoux. 1980. Neutron incoherent scattering law for diffusion in a potential of spherical symmetry: general formalism and application to diffusion inside a sphere. *Mol. Phys.* 41:271–279.

Walser, R., and W. F. van Gunsteren. 2001. Viscosity dependence of protein dynamics. *Proteins.* 42:414–421.

Williams, G., and D. C. Watts. 1970. Non-symmetrical dielectric relaxation behaviour arising from simple empirical decay function. *Trans. Faraday Soc.* 66:80–83.

Zaks, A., and A. M. Klibanov. 1984. Enzymatic catalysis in organic media at 100°C. *Science.* 224:1249–1251.