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ARTICLE

Conditioning action of the environment on the protein dynamics studied through elastic neutron scattering

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Abstract The dynamics of lysozyme in the picosecond timescale has been studied when it is in dry and hydrated powder form and when it is embedded in glycerol, glycerol–water, glucose and glucose–water matrices. The investigation has been undertaken through elastic neutron scattering technique on the backscattering spectrometer IN13. The dynamics of dry powder and embedded-in-glucose lysozyme can be considered purely vibrational up to 100 K, where the onset of an anharmonic contribution takes place. This contribution can be attributed to the activation of methyl group reorientations and is described with an Arrhenius trend. An additional source of anharmonic dynamics appears at higher temperatures for lysozyme in hydrated powders and embedded in glycerol, glycerol–water and glucose–water matrices. This second process, also represented with an Arrhenius trend, corresponds to the so-called protein dynamical transition. Both the temperature where such a transition takes place and the magnitude of the protein mean square displacements depend on the environment. The dynamical response of the protein to temperature is put in relationship with its thermal stability.

Introduction

An increasing attention is currently devoted to the study of the intriguing properties of biomolecules when they are in the presence of organic solvents (Klibanov 2001; Williams et al. 1995). In the past the features of proteins have been principally investigated when they are solved with water, which is the classic physiological milieu, while it is now becoming more and more clear that some crucial properties, such as thermal and chemical stability, can be greatly improved by properly choosing their molecular environment (Bell et al. 1995; Klibanov 2001; Hill et al. 2005). In this respect, one can roughly distinguish two different classes of media, i.e. plasticizers and stabilizers. The former class is made of compounds that enhance the protein flexibility (Gregory 1995; Tsai et al. 2000; Paciaroni et al. 2002). In terms of the well-known Conformational Substates theory, plasticizers allow the jumping among different nearly isoenergetic wells of the protein potential energy hypersurface (Frauenfelder et al. 1991). Indeed, water is the archetype of plasticizers, as it is crucial to activate the dynamics of biomolecules on wide temporal and spatial ranges (Doster et al. 1989; Gregory 1995). On the other hand, stabilizers prevent protein to jump between the conformational substates, thus inhibiting the relative

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dynamical processes (Cordone et al. 1999; Gottfried et al. 1996). From the dynamical point of view, proteins solvated with stabilizers show a sensibly slowed relaxational dynamics also at physiological temperatures (Austin et al. 1975; Hagen et al. 1995; Caliskan et al. 2004) and a significantly reduced molecular mobility (Tsai et al. 2000). Polyols, in particular sugars, are well-known stabilizers, whose properties have been often exploited to preserve structure and functionality of biomolecules in food, biotechnological and pharmaceutical sciences (Wolkers et al. 2001; Brumfiel 2004; Hill et al. 2005). For instance, proteins used for therapeutic purposes are often prepared in the solid state for reconstitution as parental solutions, as injectable suspensions, or as dry powders for inhalation (Wang 2000; Carpenter et al. 2002; Lee 2002). Biological damages caused by potentially detrimental freezing, drying and heating processes can be avoided by embedding biomolecules and cells within simple carbohydrate compounds (Burke 1986; Crowe et al. 1996, 1998). In fact, it has been suggested that such a stabilization likely proceeds through the formation of a glassy matrix of high viscosity and low mobility around the protein surface (Green and Angell 1989; Buitink and Leprince 2004). The prevalence of a plasticizer or a stabilizer component has the effect of, respectively, decreasing or increasing the glass transition temperature of the matrix surrounding the biomolecule (Green and Angell 1989; Rasmussen and MacKenzie 1971). This is supposed to be related to the corresponding decrease or increase of the bioprotective action of the matrix (Green and Angell 1989). However, it has been observed that the interaction of the matrix with the protein surface groups via direct hydrogen bond is an additional factor to take into account in determining its bioprotective effectiveness (Allison et al. 1999; Carpenter and Crowe 1988). Even though a number of studies have been performed to investigate how plasticizer and stabilizer media affect the protein structure (Liao et al. 2002), the overall scenario is incomplete without the understanding of the way they influence the protein dynamics. Many efforts in the last decade were done to shed light on this matter (Cordone et al. 1999; Tsai et al. 2000; Paciaroni et al. 2002, 2003; Caliskan et al. 2002). The protein dynamics seems to be slaved to the environment (Fenimore et al. 2004; Reat et al. 2000; Caliskan et al. 2004; Paciaroni et al. 2002), even if this point is still debated (Weik et al. 2005; Parak and Frauenfelder 1993; Kleinert et al 1998). More in detail, bulk-solvent and hydration shell fluctuations would determine, respectively, large-scale protein motions, similar to the α-relaxations of glasses, and fast protein fluctuations, reminiscent of the β-relaxations of glasses (Fenimore et al. 2002, 2004). These motions are attributed to the dynamical regime that affords proteins the flexibility for biological activity (Fenimore et al. 2004) but which also develops into the large-scale collective motions that lead to high-T unfolding (Tsai et al. 2000, 2001; De Francesco et al. 2004). On the other hand, the low-temperature dynamical response of proteins and their stability seem to be related to the degree of fragility of the glassy matrices where they are embedded (Magazù et al. 2004; Caliskan et al. 2004). Very recently, a new insight on the intimate coupling of the protein with the surrounding environment has been provided with the discovery of a quantitative relationship between the local protein relaxational dynamics and the bulk viscosity of the enclosing glassy matrices (Cornicchi et al. 2005).

As neutron scattering spectroscopy is very sensitive to the single-particle motions of hydrogen atoms (Bée 1988), such a technique gives invaluable information on the dynamical behaviour of proteins, where hydrogen atoms are abundantly and almost homogeneously distributed, in the nano- and picosecond time window over the Angstrom spatial scale (Smith 1991). Here we report a neutron scattering investigation on the molecular mobility of lysozyme in the presence of different environments, such as water, glycerol and glucose. In these media, where lysozyme is active and stable (Rariy and Klibanov 1997; Lamy et al. 1990), we characterized the extent of the protein mean square displacements (MSD) and we were able to carefully quantify the variation of the protein dynamical response to temperature and water content changes.

Materials and methods

Samples' preparation

Salt-free lyophilized lysozyme from chicken egg white has been purchased from Sigma-Aldrich (St. Louis, MO). As deuterium has a neutron scattering cross-section much lower than hydrogen (Bée 1988), we maximized the signal from the protein relative to the solvent by using only fully deuterated glucose (Euriso-Top, Saclay, France), deuterated glycerol (Sigma-Aldrich) and heavy water (Sigma-Aldrich) in samples' preparation.

In order to avoid the exchange of protons between enzyme and surrounding environment, the exchangeable hydrogens of the protein have been D-substituted by leaving in solution 1 g of lysozyme in 20 ml of D_2O at room temperature for 1 day. Using this procedure we can affirm that the spurious contribution to the incoherent quasielastic scattering due to the proton exchanging with the solvent is less than 1% of the total signal (Gregory and Lumry 1985). The solution was then freeze-dried into a powder and further dehydrated under vacuum in the presence of phosphorus pentoxide (P_2O_5) for several hours reaching a water content of a few percent.

The embedded-in-glycerol and embedded-in-glucose lysozyme samples were prepared by dissolving the freeze-dried protein and an equal weight of additive in D_2O and by lyophilizing them again to obtain a mixture of dry lysozyme + glycerol and lysozyme + glucose in proportion in weight 1:1.

The samples were subsequently hydrated by putting them in the presence of a KCl saturated solution of D_2O and varying the equilibration time. The water content w (w = g D_2O/g lysozyme) was determined by weighing the sample before and after the hydration process. We recall that in the samples with a deuterated excipient (glycerol or glucose) the quantity w denotes equivalently the ratio g D_2O/g excipient, as lysozyme and glycerol or glucose are present in the proportion in weight 1:1. With such a procedure we obtained the following samples: lysozyme powder at $0w$ and $0.4w$; lysozyme in deuterated glycerol at $0w$ and $0.4w$; lysozyme in deuterated glucose at $0w$ and $0.4w$.

Incoherent neutron scattering

Thermal neutron scattering is a powerful tool to directly probe protein internal motions. In a neutron scattering experiment the relevant quantity is the dynamical structure factor $S(Q, E)$, which represents the probability for an incident neutron to be scattered by the sample with energy and momentum transfer $E = \hbar\omega$ and $\hbar Q$, respectively. $S(Q, E)$ is the Fourier transform of the time-correlation function of the density fluctuation in the system, which provides information on both the structure and the dynamics of the investigated system through its Q- and E-dependence. In our experiments, the dynamical structure factor depends only on the modulus of the momentum transfer, as our samples are isotropic. Moreover, the dominant contribution to the revealed signal is due to non-exchangeable protein hydrogen atoms, whose neutron cross-section is by far higher than that of any other atom, and is almost exclusively incoherent in nature (σ_{inc} = 79.90 barns, σ_{coh} = 1.76 barns) (Bée 1988).

In our measurements the neutron-scattered intensity has been recorded within a narrow energy channel of 2 μeV centred at the nominal value of the elastic peak ($E \approx 0$). This channel corresponds to the energy window that we took as representative of the elastic peak and should not be confused with the energy resolution that has usually a larger value (see below). In the incoherent approximation it can be described by the law (Bée 1988; Paciaroni et al. 2002):

$$S(Q, E \approx 0) \approx \left[\mathrm{e}^{-\langle u^2 \rangle_G Q^2} A_0(Q)\delta(E) \right] \otimes L_{\mathrm{diff}}(Q, E) \otimes R(Q, E) \qquad (1)$$

The expression within square brackets describes the contribution of protein internal motions to the elastic peak. In general, such motions also give rise to a broadening of the elastic peak, and a finite sum of quasielastic structure factors multiplied by properly normalized Lorentzian functions must be added to Eq. 1 (Paciaroni et al. 2002). However, we neglected the signal from the relevant quasielastic term, within the hypothesis that it is much broader than the energy resolution. The Gaussian term is the so-called Debye–Waller factor, which describes the Q-dependence of the elastic intensity due to the vibrational atomic MSD $\langle u^2 \rangle_G$. $A_0(Q)$ is the elastic incoherent structure factor (EISF), which represents the space-Fourier transform of the scatterers distributions taken at infinite time, averaged over all the possible initial positions. Through its characteristic Q-dependence, information on geometry and type of motion of the scatterers can be obtained. We schematized the dynamics of protein hydrogen atoms with the so-called double-well jump model. Such model is an oversimplified description of the complex protein energy landscape. Nevertheless, it has been successfully employed in describing the picosecond dynamics of protein powders in different environments, such as water (Doster et al. 1989; Paciaroni et al. 2005) and glycerol (Paciaroni et al. 2002). Within this framework, the protein protons are considered dynamically equivalent and their motions are schematized as jumps between two distinct sites with a free energy difference ΔG. The corresponding EISF is

$$A_0(Q) = 1 - 2p_1p_2\left(1 - \frac{\sin(Qd)}{Qd}\right), \qquad (2)$$

where d is the spatial distance between the two potential wells, and p_1 and p_2 are the occupation probabilities of the ground and the excited state, respectively. The internal motions are convoluted with the term $L_{\mathrm{diff}}(Q, E)$, which takes into account for the global translational and rotational diffusion of the protein as a whole in the surrounding environment, and with the experimental resolution function $R(Q, E)$. This function can be approximated, in the narrow energy interval where our data were acquired, by a

Q-independent Gaussian function $R(E) = \exp(-\pi E^2/4\sigma^2)/2\sigma$, where the half-width at half-maximum (HWHM) is $\Gamma_R = 2\sigma \ln 2/\sqrt{\pi}$. In Eq. 1 we have hypothesized that internal and global diffusive motions are essentially independent, as the former are much faster than the latter. The protein global diffusion, whose contribution may broaden the measured elastic peak (Gabel 2005), in Eq. 1 has been approximated with the Fick law, thus $L_{\text{diff}} = DQ^2/\pi(E^2 + (DQ^2)^2)$. With this expression both the translational and rotational diffusion are taken into account, since rotation produces only a slight broadening of the translational contribution (Pérez et al. 1999). Actually the Fick law, which has been already exploited to take into account for the global motions of lysozyme in glycerol–water mixtures (Paciaroni et al. 2002), is only a crude approximation, since the protein moves in a very crowded environment. As a matter of fact, D is an effective self-diffusion coefficient that describes, on average, the motion of the protein as a whole, through the surrounding medium. On such grounds, all the convolution products in Eq. 1 can be done to give rise to the relationship:

$$S(Q, E \approx 0) \approx \frac{e^{-\langle u^2\rangle_G Q^2}}{2\sigma} A_0(Q) \exp\left[\frac{\pi D^2 Q^4}{4\sigma^2}\right] \times \operatorname{erfc}\left[\frac{\sqrt{\pi} D Q^2}{2\sigma}\right], \quad (3)$$

which has been used to reproduce the collected data and to derive all the parameters of the fit. In principle, to correctly evaluate the broadening of the elastic peak due to protein global motions in the present samples, one should perform accurate quasielastic measurements. However, taking into account for the high viscosity of the mixtures, the very important protein concentration, and the dynamic range of the IN13 spectrometer, we expect that this broadening is negligible, unless for lysozyme in glycerol at high w values, where samples appear to be liquid-like. In fact, we verified by the fitting procedure that the protein global diffusion gives a non-negligible contribution only for the lysozyme in glycerol at 0.4w sample.

From Eq. 3 the total and global MSD of protein H atoms can be derived via the relationship:

$$\langle u^2\rangle = -\left[\frac{d \ln S(Q, E \approx 0)}{d(Q^2)}\right]_{Q=0} = \langle u^2\rangle_G + \frac{1}{3} p_1 p_2 d^2 + \frac{D}{\pi\sigma} = \langle u^2\rangle_{\text{tot}} + \frac{D}{\pi\sigma} \quad (4)$$

In this equation, $\langle u^2\rangle_G$. is the Gaussian vibrational MSD term, while $p_1 p_2 d^2/3 = \langle u^2\rangle_c$ is the conformational contribution to MSD (Fenimore et al. 2004) and quantifies the proton mobility due to jumping between the two energetic sites. $\langle u^2\rangle_{\text{tot}}$ denotes the total contribution due to the protein internal dynamics, which is the main subject of this paper. Finally, the third term $D/\pi\sigma$ accounts for the global mobility of lysozyme.

Neutron scattering experiment

The measurements were performed on the high-resolution backscattering spectrometer IN13, at the Institut Laue-Langevin in Grenoble (France). The energy resolution of $\Gamma_R = 4.5$ μeV (HWHM) makes accessible motions faster than about 150 ps. The data were collected in the wide Q-range 0.3–4.5 Å^{-1}, with an average wave-vector transfer resolution of ~0.2 Å^{-1}. The sample holder, a standard flat aluminium cell with inner thickness of 1 mm, was placed at an angle of 135° with respect to the incident beam direction. All the samples were investigated from 20 to 320 K, acquiring the data for 1.5–2.5 h at each temperature. The acquired data were corrected in order to take into account for incident flux, cell scattering, self-shielding and detector responses. Finally, the elastic intensity of each sample relative to a given temperature was normalized with respect to the data collected at the lowest measured temperature (20 K). Since an average transmission of about 91% was obtained, we neglected the multiple scattering contribution.

Results and discussion

As we said above, in the present experiment we measured the elastic neutron scattering intensity $S(Q,E \approx 0)$ as a function of temperature for all the investigated samples. In Fig. 1a, b we show such an intensity for dry and hydrated samples at 150 K. The logarithmic scale versus Q^2 emphasizes the slight, but nevertheless non-negligible, non-Gaussian trend of $S(Q,E \approx 0)$ even at such a low temperature. The inset of Fig. 1a shows that a non-Gaussian curve (Eq. 3) reproduces better the experimental data than a Gaussian trend, with a resulting slightly lower χ^2 statistical value. Indeed the non-Gaussian character is by far more manifest at 300 K, as it is shown in Fig. 2a, b. The occurrence of anharmonic motions may be at the origin of this non-Gaussian trend which, as expected, is even more visible at room temperature in the hydrated samples (see Fig. 2b). Recent molecular dynamics simulation (Hayward and Smith 2002), NMR (Lee and Wand 2001) and neutron scattering (Roh et al. 2005) studies showed that at low temperature as well is present a non-negligible anharmonic contribution in

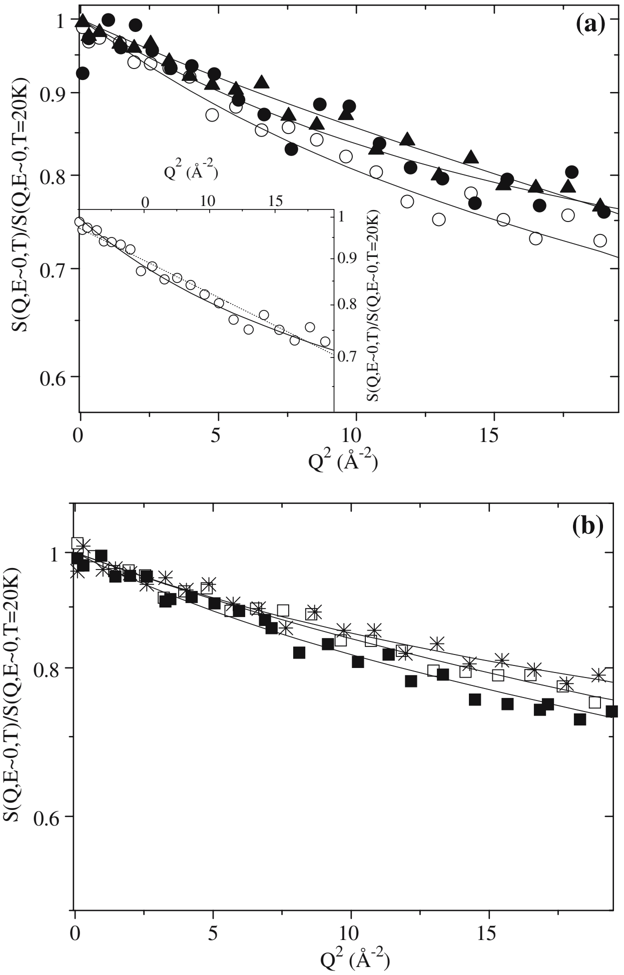

Fig. 1 Normalized incoherent elastic intensities versus Q^2 at $T = 150$ K. Panel **a**: dry sample, i.e. lysozyme powder at 0*w* (*empty circle*), lysozyme in glycerol at 0*w* (*full triangle*) and lysozyme in glucose 0*w* (*full circle*). *Inset*: normalized incoherent elastic intensity for lysozyme powder at 0*w* and the corresponding fit with Eq. 3 (*continuous line*) of the text and a Gaussian curve (*dashed line*). Panel **b**: hydrated sample, i.e. lysozyme powder at 0.4*w* (*full square*), lysozyme in glycerol at 0.4*w* (*empty square*) and lysozyme in glucose 0.4*w* (*star*). *Solid lines* are fits to Eq. 3

the protein dynamics, which comes principally from reorientation motions of methyl groups (Roh et al. 2005; Lee and Wand 2001). In fact, methyl-bearing side-chains motion makes a significant contribution to the lysozyme neutron spectra, because ~26% of all the non-exchangeable H atoms in lysozyme are on methyl groups. In this picture, purely vibrational motions take place only for temperatures up to approximately 100 K. Therefore, when we applied Eq. 3 to fit the measured elastic intensities, we estimated the purely vibrational Gaussian contribution with a linear trend, whose slope has been evaluated from the behaviour of $\langle u^2(T)\rangle_G$ versus T till 100 K. We remark that, with this new approach, the values of the double-well model parameters and the very total MSD can be somewhat different from the values already calculated approximating the low-temperature MSD trend with a hyperbolic cotangent up to ~200 K, within the framework of the Einstein model for an ensemble of harmonic oscillators (Paciaroni et al. 2002, 2005). The fitting procedure directly provides the occupation probabilities ratio p_2/p_1 as a function of T, which quantifies the ability of mobile protein protons to jump from the ground to the excited state, by overcoming the energy barrier of the parabolic potential well in which they are trapped at low temperatures. In Fig. 3 we see that the trend of p_2/p_1 for both lysozyme anhydrous powder and lysozyme embedded in glucose can be well described with a single Arrhenius function $p_2/p_1 \approx \exp(-\Delta G_{LT}/RT)$ starting from around 100 K up to room temperature, where $\Delta G_{LT} = \Delta H_{LT} - T\Delta S_{LT}$ is the free energy change due to the jump. This Arrhenius trend describes the anharmonic onset likely related to methyl groups' motions. A value of $\Delta H_{LT} = 4.0 \pm 1.0$ KJ/mol is found for both the samples, while the excess entropy $\Delta S_{LT}/R$ increases from 0.8 ± 0.3 to 1.8 ± 0.3 when we consider, respectively, lysozyme embedded in glucose and dry lysozyme samples. On the other hand, the p_2/p_1 ratio of the lysozyme solvated in glycerol sample shows the existence of two different Arrhenius-like trends. The first one, also related to the methyl group activation, is found in the low-temperature region above ~100 K and is characterized by a ΔH_{LT} quite similar to dry lysozyme powder and lysozyme embedded in glucose and slightly different $\Delta S_{LT}/R$ (see Table 1). The weak, but significant, dependence of $\Delta S_{LT}/R$ on the environment, shown in Table 1, implies that the temperature response of the methyl groups' anharmonic dynamics can be affected by changing the environment, as it will be discussed below. At higher temperatures p_2/p_1 begins to deviate from this low-T curve, then a second Arrhenius trend $\exp(-\Delta G_{HT}/RT)$, with $\Delta G_{HT} = \Delta H_{HT} - T\Delta S_{HT}$, is needed to represent the ratio up to 320 K. This second anharmonic process corresponds to the protein dynamical transition. Such a phenomenon consists in a rather sudden onset of the protein anharmonic dynamics, which takes place at a certain temperature T_d, which is around 180–200 K in hydrated proteins (Doster et al. 1989) and is a function of the external environment composition (Paciaroni et al. 2002). Such an anharmonic dynamics would be, in turn, responsible for the activation of the biological functionality (Rasmussen et al. 1992; Ferrand et al. 1993; Roh et al. 2005). The protein dynamical transition has been observed in both molecular dynamics simulation studies (Smith 1991; Vitkup et al. 2000) and

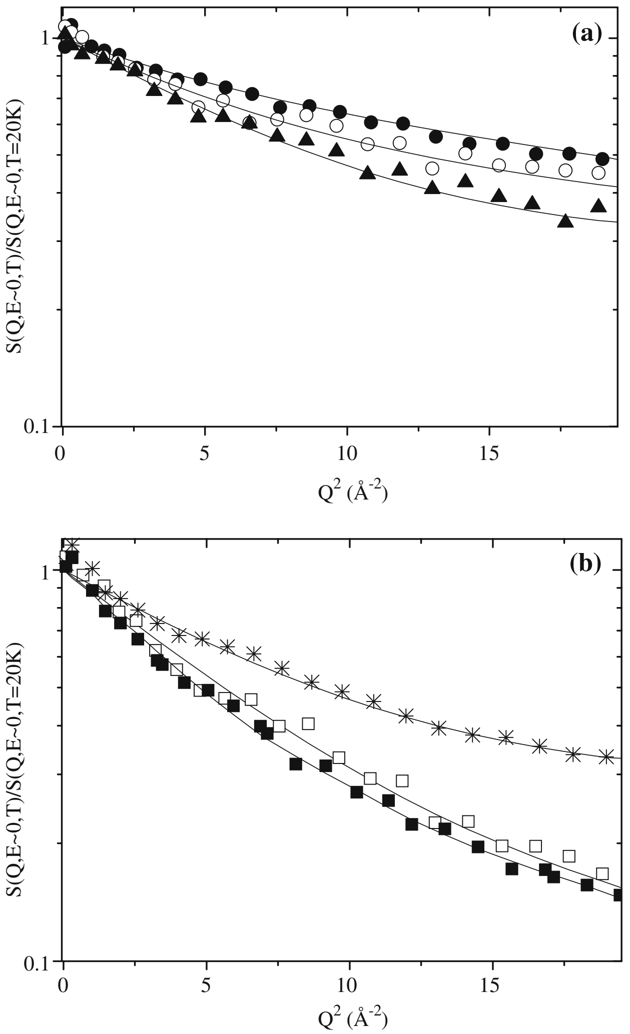

Fig. 2 Normalized incoherent elastic intensities versus Q^2 at $T = 300$ K. Panel **a**: dry sample, i.e. lysozyme powder at 0*w* (*empty circle*), lysozyme in glycerol at 0*w* (*full triangle*) and lysozyme in glucose 0*w* (*full circle*). Panel **b**: hydrated sample, i.e. lysozyme powder at 0.4*w* (*full square*), lysozyme in glycerol at 0.4*w* (*empty square*) and lysozyme in glucose 0.4*w* (*star*). *Solid lines* are fits to Eq. 3

experiments performed with many techniques such as Mossbauer (Parak et al. 1981), X-ray (Ringe and Petsko 1985), and neutron scattering (Doster et al. 1989). In dehydrated samples and when the protein is embedded in quite rigid environments, such as a sugar matrix, the dynamical transition is not visible, while it has been revealed in a softer environment such as glycerol (Tsai et al. 2000; Paciaroni et al. 2002). This is consistent with the results of Fig. 3. In this respect, the temperature at which the p_2/p_1 ratio deviates from the first Arrhenius trend is operatively defined as the dynamical transition temperature T_d. The enthalpy and the entropy changes we estimated for the second Arrhenius trend are in general much higher than the low-temperature values (see Table 1), thus suggesting that they are related to the activation of a dynamical process quite distinct from methyl group reorientation.

As we see in Fig. 4, also the p_2/p_1 ratio of hydrated samples shows two different low- and high-temperature Arrhenius trends that can be interpreted, as in the case of lysozyme solvated with glycerol, in terms of methyl group-based and dynamical transition-activated additional degrees of freedom, respectively. The ΔH_{LT} and ΔS_{LT} values are rather independent of the environment. On the other hand, when we examine the thermodynamic parameters characterizing the anharmonic activation corresponding to the dynamical transition for lysozyme in glucose–water, lysozyme in glycerol–water and hydrated lysozyme, the relative variation of ΔS_{HT} is larger than that of ΔH_{HT}. This implies that the increase of the number of configurations accessible to H atoms via jumps from the ground

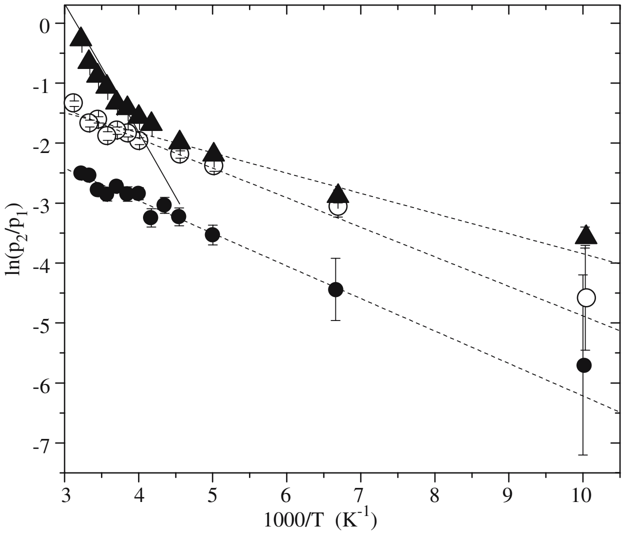

Fig. 3 Occupation probabilities ratio p_2/p_1 for lysozyme at 0*w* (*empty circle*), lysozyme in glycerol at 0*w* (*full triangle*) and lysozyme in glucose at 0*w* (*full circle*) with *dashed* and *solid lines* representing the Arrhenius-like trends as described in the text

Table 1 Parameters of the low- and high-temperature anharmonic Arrhenius processes, ΔH_{LT}, $\Delta S_{LT}/R$ and ΔH_{HT}, $\Delta S_{HT}/R$, respectively, for the different samples

	ΔH_{LT} (KJ/mol)	$\Delta S_{LT}/R$	ΔH_{HT} (KJ/mol)	$\Delta S_{HT}/R$
Lys 0*w*	4 ± 1	1.8 ± 0.3	–	–
Lys in glucose 0*w*	4 ± 1	0.8 ± 0.3	–	–
Lys in glycerol 0*w*	3 ± 1	1.0 ± 0.3	18 ± 1	6.9 ± 0.5
Lys 0.4*w*	4 ± 1	1.0 ± 0.3	16 ± 1	6.6 ± 0.5
Lys in glucose 0.4*w*	3 ± 1	0.8 ± 0.3	14 ± 1	4.8 ± 0.5
Lys in glycerol 0.4*w*	4 ± 1	1.0 ± 0.3	15 ± 1	5.5 ± 0.5

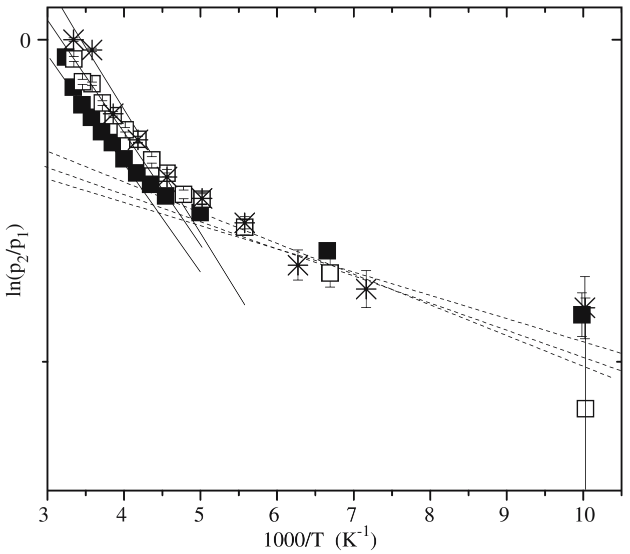

Fig. 4 Occupation probabilities ratio p_2/p_1 for lysozyme at 0.4*w* (*star*), lysozyme in glycerol at 0.4*w* (*empty square*) and lysozyme in glucose at 0.4*w* (*full square*) with *dashed* and *solid lines* representing the Arrhenius-like trends as described in the text

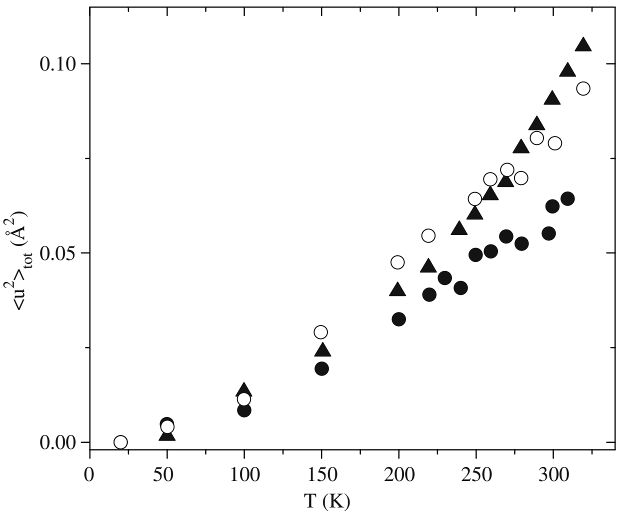

Fig. 5 Total mean square displacements versus T for lysozyme at 0*w* (*empty circles*), lysozyme in glycerol at 0*w* (*full triangle*) and lysozyme in glucose 0*w* (*full circle*)

to the excited state plays a major role with respect to the raise of the barrier between the two states. The values of ΔH_{HT} which we found for hydrated samples are in agreement with those reported by Fenimore et al. (2004) for different proteins and are attributed to β-relaxations driven by the solvent occupying the first hydration shell. In the framework of the double-well model we used, the value of $d = 1.1 \pm 0.2$ Å we found, which is nearly constant as a function of the temperature for all the measured samples, is an estimate of the mean spatial extent over which protein relaxations take place. This value can be related to side-chains torsional motions, either involving or not involving methyl groups (Doster et al. 1989; Paciaroni et al. 2002).

In Fig. 5 we show the protein total MSD of the three dry samples. Apart from a common vibrational trend up to 100 K, the mobility of dry lysozyme is definitely higher than that of lysozyme embedded in the glucose matrix, consistent with the constraining role of the sugar molecular network surrounding the protein surface. The case of lysozyme in glycerol is different, as lysozyme motions seem inhibited up to $T_d \approx 235$ K and then increase, likely due to the action of the matrix which becomes able to sustain fast relaxations (Paciaroni et al. 2002). The trend of the protein MSD for the hydrated samples, reported in Fig. 6, is instead more regular. The mobility of the lysozyme atoms progressively grows when we increase the plasticizing character of the surrounding matrix, i.e. when we consider lysozyme in glucose-water, lysozyme in glycerol-water and hydrated lysozyme. At the same time, the dynamical transition temperature shows a remarkable decrease (see also Table 2). This result confirms that around the physiological temperatures the protein dynamics can be modulated by properly choosing the plasticizing or stabilizing degree of the enclosing environment.

Additional information to characterize the protein average flexibility in the anharmonic regime is provided by evaluating the temperature dependence of the protein configurational picosecond fluctuations, as represented by the contribution $\langle u^2 \rangle_c$ in Eq. 4. At sufficiently high temperatures and sufficiently small energy resolutions, the hydrogen atoms explore the

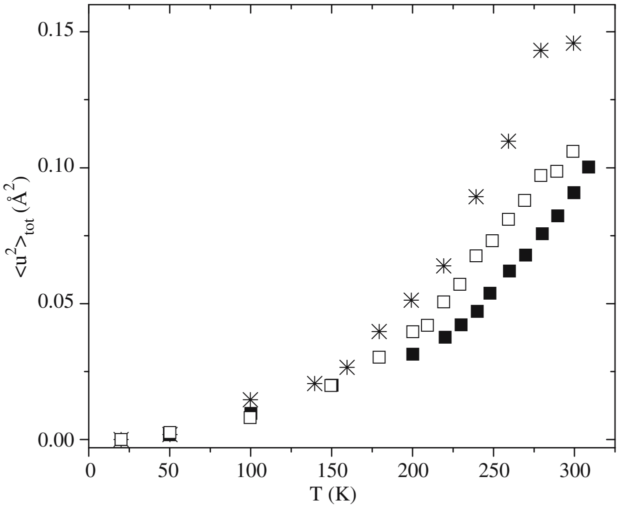

Fig. 6 Total mean square displacements versus T for lysozyme at 0.4*w* (*star*), lysozyme in glycerol at 0.4*w* (*empty square*) and lysozyme in glucose 0.4*w* (*full square*)

Table 2 Dynamical transition temperatures, rigidity, and melting transition temperatures for the measured samples

	T_d (K)	b (N/m)	T_m (K)
Lys 0*w*	–	1.8 ± 0.1	428
Lys in glucose 0*w*	–	2.9 ± 0.2	420
Lys in glycerol 0*w*	235	0.78 ± 0.03	371
Lys 0.4*w*	170	0.46 ± 0.05	347
Lys in glucose 0.4*w*	220	0.67 ± 0.02	372
Lys in glycerol 0.4*w*	205	0.62 ± 0.02	356

The first two quantities have been estimated from the data presented in the paper, while the T_ms are from Bell et al. (1995) for powders and lysozyme solvated in glycerol. The T_m of lysozyme in glucose at 0.4*w* sample has been measured in our laboratories by differential scanning calorimetry (unpublished results). The T_m of the lysozyme in glucose sample has been considered similar to that of lysozyme in sucrose (Bell et al. 1995). Lysozyme dry powder and lysozyme embedded in glucose do not show dynamical transition

entire conformational space they can access. This confining conformational space can be schematically described in terms of a harmonic average potential $V(r) = br^2/2$, so that we may write (Fenimore et al. 2004; Paciaroni et al. 2005):

$$\langle u^2 \rangle_c = \frac{k_B T}{3b} \quad (5)$$

It should be remarked that the potential used to schematize the energy landscape of protein hydrogen atoms is only apparently harmonic, as actually it consists of multiple minima with nearly equal energy and, consistent with the Conformational Substates theory (Frauenfelder et al. 1991), anharmonic motions represent jumps among such minima. The *b* parameter gives a measure of the protein rigidity, as it quantifies the linear response of protein fast fluctuations versus *T*. We remark that the *b* values calculated from Eq. 5 are dependent on the instrumental energy resolution, as the change of the accessible time-window can modify the amplitudes of the extracted MSD (Daniel et al. 1999). If Eq. 5 is used to describe the $\langle u^2 \rangle_c$ trend above the protein dynamical transition, *b* can be directly calculated. A similar approach, where the protein dynamical features are described in terms of an effective force constant, has been already proposed elsewhere (Zaccai 2000; Tehei et al. 2001). To provide new insights on the connection between the protein dynamics and the biomolecular thermal stability, in Table 2 we report the T_d and the *b* values we estimated, together with the melting temperature T_m of the measured samples, which may quantify the protein thermal stability. On a qualitative basis we may observe that higher T_d and *b* values correspond to higher T_ms. This suggests that both a retarded onset of the anharmonic motions activated with the dynamical transition (higher T_d values) and a slower response as a function of the temperature of such kind of dynamics (higher *b* values) can be crucial features to increase the protein thermal stability. This result is coherent with the picture of protein unfolding as determined by large-amplitude protein thermal fluctuations incoming above T_d (Tsai et al. 2000; Tang and Dill 1998; De Francesco et al. 2004).

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References

Allison SD, Chang B, Randolph TW, Carpenter JF (1999) Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch Biochem Biophys 365:289–298

Austin RH, Beeson KW, Eisenstein L, Frauenfelder H, Gunsalus I C (1975) Dynamics of ligand binding to myoglobin. Biochemistry 14:5355–5373

Bée M (1988) Quasielastic neutron scattering. Adam Hilger (ed), Bristol and Philadelphia

Bell LN, Hageman MJ, Muraoka LM (1995) Thermally induced denaturation of lyophilized bovine somatotropin and lysozyme as impacted by moisture and excipients. J Pharm Sci 84:707–712

Brumfiel G (2004) Just add water. Nature 428:14–15

Buitink J, Leprince O (2004) Glass formation in plant anhydrobiotes: survival in the dry state. Cryobiology 48:215–228

Burke M J (1986) The glassy state and survival of anhydrous biological systems. In: Leopold AC (ed) Membranes, metabolism and dry organisms. Cornell University Press, Ithaca pp 358–363

Caliskan G, Kisliuk A, Tsai AM, Soles CL, Sokolov AP (2002) Influence of solvent on dynamics and stability of a protein. J Non-Cryst Solids 307–310:887–893

Caliskan G, Mechtani D, Roh JH, Kisliuk A, Sokolov AP, Azzam S, Cicerone MT, Lin-Gibson S, Peral I (2004) Protein and solvent dynamics: how strongly are they coupled? J Chem Phys 121:1978–1983

Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25:244–255

Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW (2002) Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol 13:109–133

Cordone L, Ferrand M, Vitrano E, Zaccai G (1999) Harmonic behavior of trehalose-coated carbon-monoxy-myolobin at high temperature. Biophys J 76:1043–1047

Cornicchi E, Onori G, Paciaroni A (2005) Picosecond-time-scale fluctuations of proteins in glassy matrices: the role of viscosity. Phys Rev Lett 95:158104(1)–158104(4)

Crowe LM, Reid DS, Crowe JH (1996) Is trehalose special for preserving dry biomaterials? Biophys J 71:2087–2093

Crowe JH, Carpenter JF, Crowe LM (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73–103

Daniel RM, Finney JL, Reat V, Dunn R, Ferrand M, Smith JC (1999). Enzyme dynamics and activity: time-scale dependence of dynamical transitions in glutamate dehydrogenase solution. Biophys J 77:2184–2190

De Francesco A, Marconi M, Cinelli S, Onori G, Paciaroni A (2004) Picosecond internal dynamics of lysozyme as affected by thermal unfolding in nonaqueous environment. Biophys J 86:480–487

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

Fenimore PW, Frauenfelder H, McMahon BH, Parak FG (2002) Slaving: Solvent fluctuations dominate protein dynamics and functions. Proc Natl Acad Sci USA 99:16047–16051

Fenimore PW, Frauenfelder H, McMahon BH, Young RD (2004) Bulk-solvent and hydration-shell fluctuations, similar to α- and β-fluctuations in glasses, control protein motions and functions. Proc Natl Acad Sci USA 101:14408–14413

Ferrand M, Dianoux AJ, Petry W, Zaccai G (1993) Thermal motions and function of bacteriorhodopsin in purple membranes: effects of temperature and hydration studied by neutron scattering. Proc Natl Acad Sci USA 90:9668–9672

Frauenfelder H, Sligar SG, Wolynes PG (1991) The energy landscapes and motions of proteins. Science 254:1598–1603

Gabel F (2005) Protein dynamics in solution and powder measured by incoherent elastic neutron scattering: the influence of Q-range and energy resolution. Eur Biophys J 34:1–12

Gottfried DS, Peterson ES, Sheikh AG, Wang J, Yang M, Friedman CA (1996) Evidence for damped hemoglobin dynamics in a room temperature trehalose glass. J Phys Chem 100:12034–12042

Green JL, Angell CA (1989) Phase relations and vitrification in saccharide–water solutions and the trehalose anomaly. J Phys Chem 93:2880–2882

Gregory RB (1995) Protein-solvent interactions. Marcel Dekker, New York

Gregory RB, Lumry R (1985) Hydrogen exchange evidence for distinct structural classesein globular proteins. Biopolymers 24:301–326

Hagen SJ, Hofrichter J, Eaton WA (1995) Protein reaction kinetics in a room temperature glass. Science 269:959–962

Hayward JA, Smith JC (2002) Temperature dependence of protein dynamics: computer simulation analysis of neutron scattering properties. Biophys J 82:1216–1225

Hill JJ, Shalaev EY, Zografi G (2005) Thermodynamic and dynamic factors involved in the stability of native protein structure in amorphous solids in relation to levels of hydration. J Pharm Sci 94:1636–1667

Kleinert T, Doster W, Leyser H, Petry W, Schwarz V, Settles M (1998) Solvent composition and viscosity effects on the kinetics of CO-binding to horse myoglobin. Biochemistry 37:717–733

Klibanov AM (2001) Improving enzymes by using them in organic solvents. Nature 409:241–246

Lamy L, Portmann MO, Mathlouthi M, Larreta-Garde V (1990) Modulation of egg-white lysozyme activity by viscosity intensifier additives. Biophys Chem 36:71–76

Lee G (2002) Spray-drying of proteins. Pharm Biotechnol 13:135–158

Lee AL, Wand AJ (2001) Microscopic origins of entropy, heat capacity and the glass transition in proteins. Nature 411:501–504

Liao YH, Brown MB, Quader A, Martin GP (2002) Protective mechanism of stabilizing excipients against dehydration in the freeze-drying of proteins. Pharm Res 19(12):1854–1861

Magazù S, Maisano G, Migliardo F, Mondelli C (2004) Mean-Square displacement relationship in bioprotectant systems by elastic neutron scattering. Biophys J 86:3241–3249

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

Paciaroni A, Orecchini A, Cinelli S, Onori G, Lechner RE, Pieper J (2003) Protein dynamics on the picosecond timescale as affected by the environment: a quasielastic neutron scattering study. Chem Phys 292:397–404

Paciaroni A, Cinelli S, Cornicchi E, De Francesco A, Onori G (2005) Fast fluctuations in protein powders: The role of hydration. Chem Phys Lett 410:400–403

Parak F, Frauenfelder H (1993) Protein dynamics. Physica A 201:332–345

Parak F, Frolov EN, Mossbauer RL, Goldanskii VI (1981) Dynamics of metmyoglobin crystals investigated by nuclear gamma resonance absorption. J Mol Biol 145:825–833

Pérez J, Zanotti J-M, Durand D (1999) Evolution of the internal dynamics of two globular proteins from dry powder to solution. Biophys J 77:454–469

Rariy RV, Klibanov AM (1997) Correct protein folding in glycerol. Proc Natl Acad Sci USA 94:13520–13523

Rasmussen DH, MacKenzie AP (1971) The glass transition in amorphous water. Application of the measurement to problems arising in cryobiology. J Phys Chem 75:967–973

Rasmussen BF, Stock AM, Ringe D, Petsko GA (1992) Crystalline ribonuclease A loses function below the dynamical transition at 220 K. Nature 357:423–424

Reat V, Dunn R, Ferrand M, Finney JL, Daniel RM, Smith JC (2000) Solvent dependence of dynamic transitions in protein solutions. Proc Natl Acad Sci USA 97:9961–9966

Ringe D, Petsko GA (1985) Mapping protein dynamics by X-ray diffraction. Prog Biophys Mol Biol 45:197–235

Roh JH, Novikov VN, Gregory RB, Curtis JE, Chowdhuri Z, Sokolv AP (2005) Onsets of anharmonicity in protein dynamics. Phys Rev Lett 95:038101(1)–038101(4)

Smith JC (1991) Protein dynamics: comparison of simulations with inelastic neutron scattering experiments. Q Rev Biophys 24:1–65

Tang KES, Dill K (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, Madern D, Pfister C, Zaccai G (2001) Fast dynamics of halophilic malate dehydrogenase and BSA measured by neutron scattering under various solvent conditions influencing protein stability. Proc Natl Acad Sci USA 98:14356–14361

Tsai AM, Neumann DA, Bell LN (2000) Molecular dynamics of solid-state lysozyme as affected by glycerol and water: a neutron scattering study. Biophys J 79:2728–2732

Tsai AM, Udovic TJ, Neumann DA (2001) The inverse relationship between protein dynamics and thermal stability. Biophys J 81:2339–2343

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

Wang W (2000) Lyophilization and development of solid protein pharmaceuticals. Int J Pharm 203:1–60

Weik M, Lehnert U, Zaccai G (2005) Liquid-like water confined in stacks of biological membranes at 200 K and its relation to protein dynamics. Biophys J 89:3639–3646

Williams DLJ, Rapanovich I, Russell AJ (1995) Proteins in essentially nonaqueous environments. In: Gregory RB (ed) Protein–solvent interactions, Marcel Dekker, New York

Wolkers WF, Walker NJ, Tablin F, Crowe JH (2001) Human platelets loaded with trehalose survive freeze-drying. Cryobiology 42:79–87

Zaccai G (2000) How soft is a protein? a protein dynamics force constant measured by neutron scattering. Science 288:1604–1607