Effect of the Environment on the Protein Dynamical Transition: A Neutron Scattering Study

Alessandro Paciaroni, Stefania Cinelli, and Giuseppe Onori
Istituto Nazionale per la Fisica della Materia, Dipartimento di Fisica dell'Università di Perugia, Perugia 06121, Italy

ABSTRACT We performed an elastic neutron scattering investigation of the molecular dynamics of lysozyme solvated in glycerol, at different water contents h (grams of water/grams of lysozyme). The marked non-Gaussian behavior of the elastic intensity was studied in a wide experimental momentum transfer range, as a function of the temperature. The internal dynamics is well described in terms of the double-well jump model. At low temperature, the protein total mean square displacements exhibit an almost linear harmonic trend irrespective of the hydration level, whereas at the temperature T_d a clear changeover toward an anharmonic regime marks a protein dynamical transition. The decrease of T_d from ~238 K to ~195 K as a function of h is reminiscent of that found in the glass transition temperature of aqueous solutions of glycerol, thus suggesting that the protein internal dynamics as a whole is slave to the environment properties. Both T_d and the total mean square displacements indicate that the protein flexibility strongly rises between 0.1 and 0.2h. This hydration-dependent dynamical activation, which is similar to that of hydrated lysozyme powders, is related to the specific interplay of the protein with the surrounding water and glycerol molecules.

INTRODUCTION

The functionality of proteins depends in a critical fashion on their ability in properly performing the conformational rearrangements necessary to carry out their specific biological action (McCammon and Harvey, 1987). A crucial contribution to such conformational changes is supposed to be made by fast stochastic structural fluctuations on the pico- and nanosecond temporal windows (Smith, 1991; Fitter et al., 1996). On these timescales, many experimental techniques (Teeter et al., 2001) and molecular dynamics simulation (Vitkup et al., 2000) have shown that proteins undergo a dynamical transition to a glass-like solid state, at a temperature T_d near 200 K. Below T_d, proteins show a practically harmonic internal dynamics, whereas above T_d, the onset of large-amplitude anharmonic motions takes place (Doster et al., 1989). It has been supposed that these motions may play a key role on increasing the intrinsic protein flexibility to achieve functional configurations (Frauenfelder and McMahon, 1998).

The existence of this dynamical transition in proteins and some essential quantitative peculiarities, such as the T_d value and the mean square displacements amplitudes, depend on both the kind and the amount of the molecules surrounding the protein surface (Gregory, 1995). These molecules make an environment that may act as either plasticizer or stabilizer by respectively allowing or preventing protein to jump between the so-called conformational substates, i.e., nearly isoenergetic wells of its potential energy hypersurface (Frauenfelder et al., 1991). Water, which is the natural medium of biomolecules, is a well-known plasticizer (Gregory, 1995). The enhancement of flexibility produced by hydration seems to be decisive in activating the internal motions that underlie the dynamical transition (Doster et al., 1989; Pissis, 1992; Gregory and Chai, 1993), which is apparently absent in the dry state (Ferrand et al., 1993). Conversely, when proteins are embedded within the glassy matrix of a stabilizer medium such as trehalose, they exhibit a purely harmonic trend, the dynamical transition being suppressed (Gottfried et al., 1996; Cordone et al., 1999). On the other hand, proteins solvated with molecules of glycerol, which has a stabilizer character too, show a sensibly slowed relaxational dynamics also at physiological temperatures (Austin et al., 1975; Tsai et al., 2001). In addition, their molecular mobility is significantly reduced (Tsai et al., 2000), even if they still exhibit a well-defined dynamical transition (Tsai et al., 2000).

Actually there is still a controversial debate on whether the dynamical transition is determined by the solvent properties (Smith, 1991; Diehl et al., 1997; Lehnert et al., 1998; Vitkup et al., 2000) or whether it is an intrinsic property of proteins (Parak and Frauenfelder, 1993). On the basis of the results mentioned above, it can be supposed that both the onset and the amplitude of the protein anharmonic motions related to the dynamical transition can be controlled by choosing suitably the physicochemical properties of the protein environment (Frauenfelder and McMahon, 1998). This perspective would have a number of practical consequences on the biological and pharmaceutical fields (Frauenfelder and McMahon, 1998; Klibanov, 2001).

On these grounds, to better understand how the protein dynamics is modulated by the interplay with its environment, we performed a detailed investigation on the molecular mobility of lysozyme solvated with glycerol, at different water contents, as a function of the temperature. In these mixtures, where lysozyme is active and stable (Rariy and

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Address reprint requests to Dr. Alessandro Paciaroni, Dipartimento di Fisica dell'Università di Perugia, Via Pascoli I-06123, Perugia 06121, Italy. Tel.: 0039-075-5852785; Fax: 0039-075-44666; E-mail: alessandro.paciaroni@fisica.unipg.it.

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Klibanov, 1997), we followed how the stabilizing action of glycerol is perturbed by the plasticizing effect of water molecules. In addition, because the glass transition temperature of aqueous solutions of glycerol is known, we could directly relate the dynamical transition of lysozyme in the glycerol-water mixtures to that of the environment.

In this paper, we will show that both the dynamical transition temperature T_d and the protein atomic mean square displacements in glycerol-based glassy mixtures are strongly dependent on the hydration degree. Quite strikingly, the protein dynamics exhibits a sharp onset between 0.1*h* and 0.2*h*. This behavior points out a remarkable analogy between the protein mobility in glycerol-water glassy mixtures and in hydrated protein powders.

MATERIALS AND METHODS

Samples

Hen egg white lysozyme was purchased from Sigma (St. Louis, MO) and used without further purification. To single out the incoherent signal from nonexchangeable protein hydrogen atoms (see below), all the samples were prepared with fully deuterated glycerol and heavy water (Sigma). In low-hydrated systems, the effects on the protein dynamics due to solvent deuteration are usually neglected (Doster et al., 1989; Fitter et al., 1997; Pérez et al., 1999; Tsai et al., 2000, 2001). An amount of lysozyme of 2 g was previously dissolved in 20 g of D_2O to properly substitute exchangeable hydrogen atoms with deuterium. After 7 days the solution was freeze-dried into a powder that was, in turn, exsiccated under vacuum in presence of P_2O_5 to achieve a water content as low as possible. Then, the protein was dissolved in a solution of D_2O plus glycerol and lyophilized again to obtain a mixture with lysozyme and glycerol in proportion in weight of 1:1, and a hydration degree of 0*h*. In the following, the lysozyme-glycerol mixture was hydrated in the presence of a saturated KCl solution of D_2O, to give rise to the samples corresponding to 0.1*h*, 0.2*h*, 0.35*h*, 0.42*h*, and 0.83*h*.

Incoherent neutron scattering

In a neutron scattering experiment on protein the molecular motions are investigated by measuring the so-called dynamical structure factor $S(Q, E)$, which gives the probability that an incident neutron is scattered by the sample with an energy transfer E and a momentum transfer hQ (Bée, 1988), where Q is the wave-vector transfer. The functional dependence of $S(Q, E)$ on E and Q provides information on, respectively, the characteristic times and the geometry of the molecular motions that give rise to the revealed signal. The dynamical structure factor, in turn, includes both coherent and incoherent contributions, which arise from, respectively, inter- or self-particle correlations of collective or individual atomic motions. The hydrogen atom is characterized by a very large, almost exclusively incoherent cross section ($\sigma_{inc} = 79, 90$ barns vs. $\sigma_{coh} = 1.76$ barns), which is by far higher than the coherent or incoherent cross section of deuterium or of any other element (Bée, 1988). Because in our samples deuterated glycerol and heavy water were used, the most significant contribution to the revealed signal is incoherent in nature, coming from the large amount of protein nonexchangeable hydrogen atoms. These atoms are, in turn, copiously and uniformly distributed throughout the whole protein, thus allowing for a complete sampling of its molecular vibrational and diffusive motions, within the time and spatial windows defined by the experimental E and Q resolutions and ranges. In addition, because all the samples are isotropic, the measured intensity will show a dependence on the only exchanged momentum modulus. In the incoherent approximation, the low-energy dynamical structure factor of protein hydrogen atoms can be described by the law (Bée, 1988):

$$S(Q, E) = \left[e^{-\langle u^2 \rangle Q^2} \left(A_0(Q)\delta(E) + \sum_{i=1}^{n} A_i(Q) L_i(Q, E) \right) \right] \otimes L_{diff}(Q, E) \otimes R(Q, E) \quad (1)$$

In this equation, the expression within the square brackets describes the protein internal, i.e., relative to its center of mass, relaxational motions. These motions are then convoluted with the term $L_{diff}(Q, E)$, which represents a possible global translational and/or rotational diffusion of the protein as a whole in the medium, and with the experimental resolution function $R(Q, E)$. In Eq. 1 the inelastic contribution does not appear, because in the low-energy transfer range explored in the present experiment the vibrational contribution is negligible. Apart from the so-called Debye-Waller factor, which describes the Gaussian Q decrease due to the vibrational atomic mean square displacements $\langle u^2 \rangle$, the spectrum arising from protein internal motions is usually distinct in two components, the elastic and the quasielastic term. Obviously, the energy dependence of the elastic contribution is given by a delta function $\delta(E)$, whereas the modulation as a function of Q is provided by the elastic incoherent structure factor (EISF) A_0. Such a factor represents the space-Fourier transform of the scatterers distribution taken at infinite time, averaged over all the possible initial positions. In the second term, the broadening of the elastic peak is described by the sum of the n different kinds of relaxations sampled by the hydrogen atoms. In Eq. 1, we suppose that each of these relaxations is represented by a quasielastic structure factor A_i, multiplied by a properly normalized Lorentzian function $L_i(Q, E)$. The elastic and the quasielastic structure factors are related through the sum rule $\Sigma_{i=0}^{n} A_i(Q) = 1$. In the present experiment the neutron-scattered intensity has been recorded within a narrow energy interval of 2 μeV centered at $E \approx 0$. In this operative condition, already employed in the past to study the dynamics of biological samples (Doster et al., 1989; Zaccai, 2000; Tsai et al., 2000; Tehei et al., 2001), we singled out only the intensity of elastically scattered neutrons $S(Q, E \approx 0)$, as a function of both Q and T. Thus, Eq. 1 becomes:

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

Actually, for Q values smaller than the inverse of the characteristic length scale of the protein atomic motions, the elastic intensity can be described through a Gaussian dependence $S(Q, E \approx 0) \approx \exp(-\langle u^2 \rangle Q^2)$, to directly derive the hydrogen mean square displacements (Zaccai, 2000; Tsai et al., 2000; Tehei et al., 2001). However, we verified that this approximation is unable to correctly reproduce the experimental data in the wide Q range we investigated, as a function of both the temperature and hydration (see below). Thus, to interpret the measured elastic intensity we have done some work hypotheses on the different components of Eq. 2. At first, to describe the protein internal motions we adopted the so-called double-well jump model, which has been successfully exploited in explaining the dynamics of hydrated myoglobin powders on the same energy resolution and Q range as the present experiment (Doster et al., 1989), with the corresponding EISF:

$$A_0^{2W}(Q) = 1 - 2p_1p_2\left(1 - \frac{\sin(qd)}{qd}\right), \quad (3)$$

where p_1 and p_2 are the probabilities of finding the hydrogen atom, respectively, in the ground and excited state, and d is the distance between the two wells. In this simplified picture, the protein hydrogen atoms, which are supposed to be dynamically equivalent, may jump between two distinct

sites of different free energy (Bée, 1988; Doster et al., 1989). On the other hand, the global diffusion has been approximated with the Fick law:

$$L_{\text{diff}} = \frac{1}{\pi}\frac{DQ^2}{E^2 + (DQ^2)^2}\ , \tag{4}$$

where D is an effective self-diffusion coefficient that describes on the average the motion of the protein as a whole, through the medium consisting of water, glycerol, and the other protein molecules. In this picture translational and rotational diffusion are both taken into account in Eq. 4, because rotation produces only a slight broadening of the translational contribution (Pérez et al., 1999). Despite this crude approximation, we obtained reasonable D values, as we shall see below.

To express Eq. 2 in a completely analytical fashion, we have fitted the experimental resolution with a Q independent Gaussian function $R(E) = \exp(-\pi E^2/4\sigma^2)/2\sigma$, where the half-width at half-maximum is $\Gamma_R = 2\sigma\ln 2/\sqrt{\pi}$. Such an approximation is quite good in the narrow energy interval, where the data were acquired. On such grounds, all the convolution products in Eq. 2 can be done to give rise to the relationship:

$$S(Q,E \approx 0) \approx \frac{e^{-\langle u^2\rangle Q^2}}{2\sigma} A_0^{2W}(Q)\exp\left[\frac{\pi D^2 Q^4}{4\sigma^2}\right] \times \operatorname{erfc}\left[\frac{\sqrt{\pi}DQ^2}{2\sigma}\right], \tag{5}$$

which has been used to reproduce the collected data. In the fitting procedure, the temperature dependence of $\langle u^2\rangle$ in the Debye-Waller factor was described in terms of a set of quantized harmonic oscillators as in an Einstein solid, through the following relationship (Smith, 1991):

$$\langle u^2\rangle = \frac{\hbar\omega}{2K}\coth\left(\frac{\hbar\omega}{2k_BT}\right) - \langle u^2\rangle_0\ , \tag{6}$$

where K and ω are, respectively, the average force field constant and the average frequency of the set of the oscillators; accordingly, the relationship $\langle u^2\rangle_0 = \hbar\omega/2K$ provides the zero-point mean square displacements. Actually, because the measured elastic intensity has been normalized with respect to the lowest temperature (see below), the zero-point mean square displacements have been subtracted in the right side of Eq. 6. The description of Eq. 6 is reasonable if we assume that $\langle u^2\rangle$ values arise from the averaged one-phonon vibrational contribution of hydrogen atoms. We checked that a more accurate approximation, which should involve the protein vibrational density of states (Bée, 1988), does not produce significant changes with respect to the behavior predicted by Eq. 6.

From Eq. 5 the total mean square displacements can be derived via the following relationship (Doster et al., 1989):

$$\langle u^2\rangle_{\text{tot}} = -\left\{\frac{d\ln(S(Q, E \approx 0)}{d(Q^2)}\right\}_{Q=0} = \langle u^2\rangle + \frac{1}{3}p_1p_2d^2\ , \tag{7}$$

where the two terms on the right side represent, respectively, the vibrational contribution described by Eq. 6 and the additional local atomic mobility of the protein internal motions described by Eq. 3. Actually, the quantity $\langle u^2\rangle_{\text{tot}}$ does not account for the global mobility expressed by Eq. 4.

Neutron scattering experiment

The measurements were performed on the high-energy resolution, wide momentum transfer backscattering spectrometer IN13, at the Institut Laue-Langevin. An energy resolution with a half-width at half-maximum Γ_R = 4.5 μeV and an average wave-vector transfer resolution of ~0.2 Å^{-1}, corresponding to an incident wavelength of 2.23 Å, were achieved in the Q range 0.3–5.3 Å^{-1}. Such an instrumental resolution function makes accessible only motions faster than $\sim\hbar/\Gamma_R \approx 150$ ps in a spatial region smaller than ~5 Å. An amount of ~0.5 g of sample was held in a standard flat aluminum cell with internal spacing of 1 mm, placed at an angle of 135° with respect to the incident beam. We investigated the samples in a temperature range from 20 K to 310 K. The data were corrected to take into account incident flux, cell scattering, self-shielding, and detector response. Then, the intensity of each sample was normalized with respect to the corresponding lowest measured temperature. Because an average transmission of 92% was obtained, multiple scattering processes have been neglected.

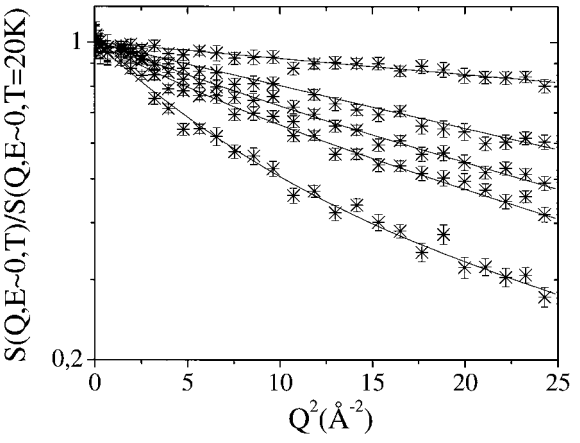

FIGURE 1 Normalized elastic neutron scattering intensity of lysozyme in pure glycerol versus Q^2, at five selected temperatures: 100, 200, 240, 260, and 300 K, from top to bottom. The lines correspond to fitting with Eq. 5.

RESULTS AND DISCUSSION

In Fig. 1 we show some typical measured elastic neutron scattering intensities, as a function of Q^2 for different measured temperatures. In particular, the curves displayed are relative to lysozyme solvated in pure glycerol, as they look after the usual standard corrections and normalization (see Materials and Methods). The logarithmic scale allows us to clearly discern that up to ~240 K, the elastic intensity exhibits a Q^2 dependence that can be satisfactorily described with a Gaussian-like Debye-Waller factor. However, just at around 240 K, the data show a slight departure from the Gaussian behavior, which becomes more and more marked as the temperature increases. Such a behavior is similar to that revealed in hydrated protein powders, where the departure from the Gaussian dependence has been put in relationship with the onset of anharmonic motions involving jumps of the hydrogen atoms in a double-well potential (Doster et al., 1989). This resemblance suggests that the spectra of lysozyme solvated in pure glycerol and those of protein hydrated powders may originate from similar dynamical processes. Actually, the elastic intensity of the sample with hydration degree 0h, is quite well described by

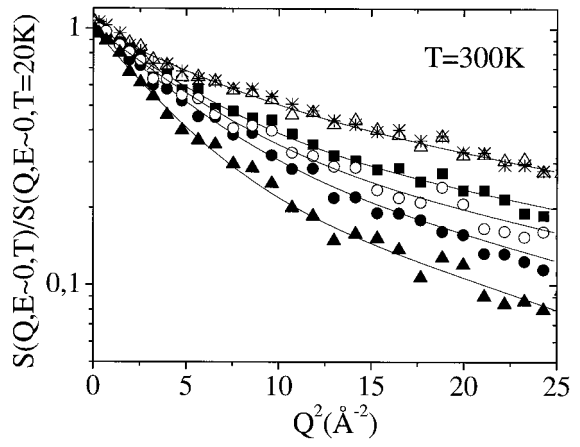

FIGURE 2 Normalized elastic neutron scattering intensity of lysozyme at 300 K vs. Q^2, for the different measured water contents: △, $h = 0$. g D_2O/g Lys; *$h = 0.1$ g D_2O/g Lys; ■, $h = 0.2$ g D_2O/g Lys; ○, $h = 0.35$ g D_2O/g Lys; ●, $h = 0.42$ g D_2O/g Lys; ▲, $h = 0.83$ g D_2O/g Lys. In all of the following figures the same notation will be used. The lines correspond to the fitting with Eq. 5.

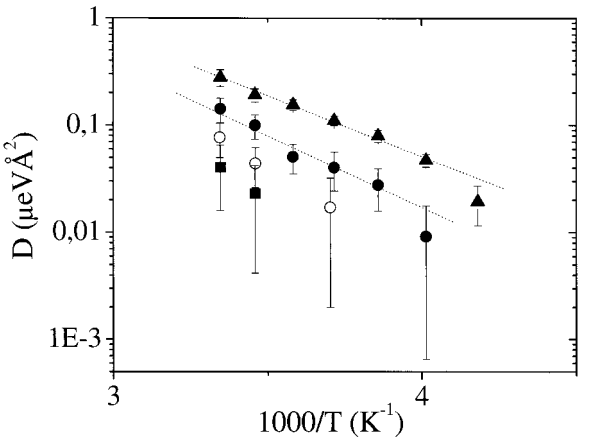

FIGURE 3 Estimated lysozyme self-diffusion coefficients versus 1000/T, for different water contents. The dashed lines correspond to the linear Arrhenius trend.

a simple double-well jump model, as shown in Fig. 1. The fitting procedure of the experimental data with Eq. 5 provides a parameter $D = 0$ μeVÅ^2, which corresponds to the absence of the global diffusion process. The distance between the two sites assumes a value of $d = 1.2 \pm 0.1$ Å, which is nearly constant as a function of the temperature. To attribute a physical meaning to such a parameter, which affects as well the total mean square displacements values (see Eq. 7), we observe that a large variety of protein internal movements occurs within the nano- and picosecond windows, such as methyl group rotations, hydrogen bond network arrangements, protein domain concerted motions, and side-chains confined diffusive dynamics (Fitter et al., 1996). Because the measured elastic intensity reflects the dynamical contribution of the protein motions that are accessible in the experimental energy and momentum transfer range, then the double-well jump model may provide only an average description of the protein internal dynamics. In this context, and by analogy of what is suggested in the case of hydrated protein powders (Doster et al., 1989; Diehl et al., 1997), our experimental data reveal the existence of a protein internal dynamics characterized by a spatial extent d, which may be related to relaxational degrees of freedom involving fast dihedral angle fluctuations, i.e., the torsion jumps of the side-chain protons.

Fig. 2 shows that, as water content increases, the drop of the elastic intensity as a function of Q^2 becomes more pronounced. Nevertheless, Eq. 5 is still able to properly describe the experimental data, but the fitting procedure provides finite values for the D coefficients, thus indicating that global diffusive motions as well may give a significant contribution. With this respect, we find that d is nearly constant as h changes, whereas the D parameters exhibit a visible dependence on both h and T, as Fig. 3 points out. The general behavior of these coefficients indicates that, as the water content diminishes, the global diffusion is more and more slowed down, thus becoming observable only at temperatures progressively increasing. In particular, we obtained finite D values only for samples with a water content higher than $\sim 0.2h$. For mixtures with $0.42h$ and $0.83h$ we may infer that the diffusion coefficients follow an Arrhenius-like trend down to ~250 K, where a slight deviation seems to occur. In conditions of infinite dilution the diffusion coefficient D_0 at 298 K can be calculated through the Stokes formula $D_0 = K_B T/6\pi\eta a$, because the protein radius a and the medium shear viscosity η of aqueous solution of glycerol are known (Pérez et al., 1999; Lide, 2001). In the mixtures under investigation, however, we expect to find sensibly lower self-diffusion coefficient values, due to the intermolecular interaction between different proteins. In particular, the ratio D_0/D, which quantifies the deviations from the infinite dilution conditions, turns out to be ~3–4 in the investigated hydration region. This result is in agreement with the theoretical estimate for D_0/D obtained for simple colloidal suspensions of hard spheres, where the short-times translational self-diffusion coefficient is described through the well-known virial expansion proposed by Beenakker and Mazur (1983). Such an agreement suggests that the approximation we made to take into account global diffusion is reasonable.

Analogously to the elastic intensity, the total mean square displacements shown in Fig. 4 provide a quantitative measure of the average hydrogen protein mobility, due to internal molecular relaxations. In general, at low T, the $\langle u^2 \rangle_{tot}$ of all the samples follow the harmonic Einstein-like behavior expressed through Eq. 6, with parameters $\langle u^2 \rangle_0 = 0.012 \pm 0.001$ Å^2 and $\omega = 11 \pm 1$ meV. However, at a certain temperature T_d, the mean square displacements show a marked deviation from the purely vibrational trend. In terms

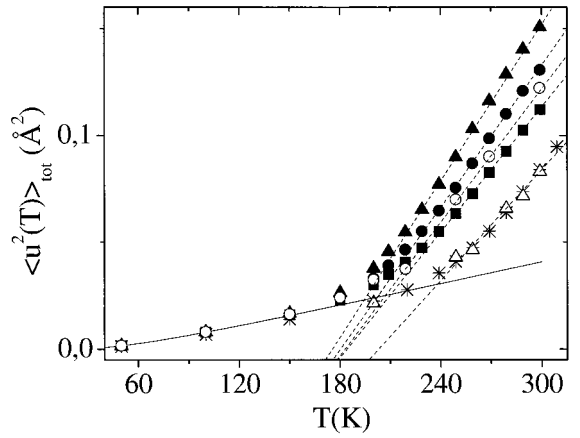

FIGURE 4 $\langle u^2\rangle_{tot}$ versus T, for all the measured water contents. The solid line represents the harmonic trend of Eq. 6. Dashed lines are linear fits to the high-temperature data.

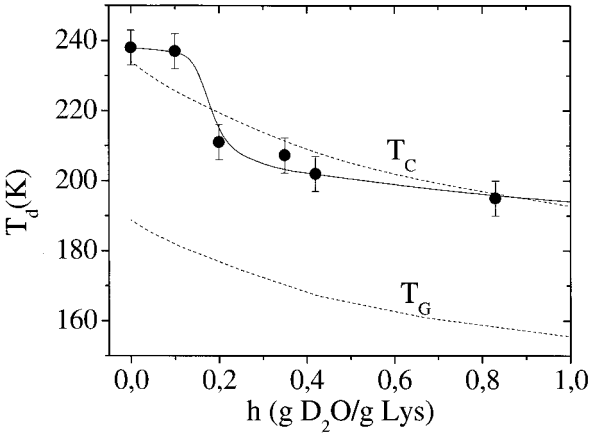

FIGURE 6 Lysozyme T_d (●) versus h, compared with the glass transition T_G and the estimated dynamical phase transition T_C temperatures (*dashed line*) of the corresponding aqueous solutions of glycerol. The solid line is a guide for the eye.

of the model we have exploited, such a deviation arises when the temperature is high enough to activate double-well jumps over the energy barrier of different conformational substates. These anharmonic processes correspond to the onset of internal structural relaxations. As shown in Fig. 4, we estimated the values of T_d as the intercept between the low-T curve (Eq. 6) and the straight lines describing $\langle u^2\rangle_{tot}$ at higher temperature. Our data show that lysozyme in glycerol undergoes a dynamical transition at around 238 K, at variance with Tsai et al. (2000), who found $T_d \approx 270$ K. We may ascribe this disagreement to the fact that in the present study we employed a different analysis, the double-well jump model, on data acquired on a spectrometer with a different dynamical range, thus sampling diverse molecular motion details.

In Fig. 5 we show the $\langle u^2\rangle_{tot}$ of lysozyme in glycerol with 0h together with the mean square displacements of partially deuterated bulk glycerol $C_3H_5(OD)_3$, also measured on the spectrometer IN13 (Wuttke et al., 1995). Because the mean square displacements of $C_3H_5(OD)_3$, which refer to backbone molecular mobility, are practically identical to those of $C_3D_5(OH)_3$ (Wuttke et al., 1995), they represent on average the mean square displacements of all the glycerol hydrogen atoms. The striking similar mobility of lysozyme and pure glycerol suggests that the protein dynamics in the anhydrous sample is strongly related to that of its environment. Fig. 5 seems to indicate that both lysozyme in glycerol and bulk glycerol undergo a dynamical transition at the same temperature. Actually, the T_d that we found for lysozyme in pure glycerol is close to the dynamical phase transition temperature obtained for pure glycerol $T_C \approx 233$ K (Franösc et al., 1997). T_C is a critical temperature below which, according to the mode coupling theory (MCT), relaxations in glass-forming systems are arrested, and spontaneous breaking of ergodicity occur (Götze, 1991). This result indicates that glycerol, despite its high viscosity, is nevertheless able to support the protein structural relaxations, but only above T_C, when glycerol itself may sustain density relaxations.

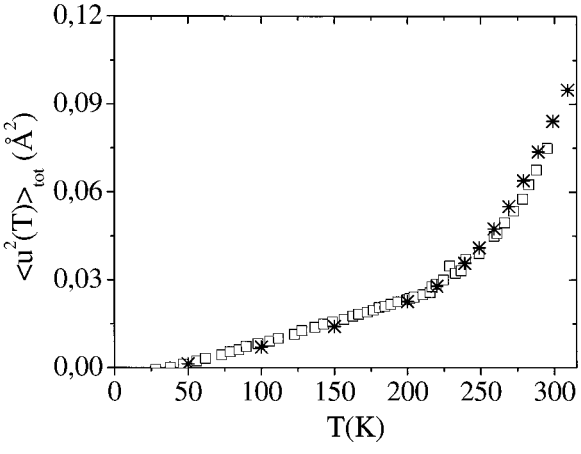

FIGURE 5 $\langle u^2\rangle_{tot}$ of lysozyme solvated with only glycerol compared with the mean square displacements of pure glycerol (□). These data were taken from Wuttke et al., 1995.

In general, the T_d values of the measured samples are strongly modulated by the water content, as shown in Fig. 6. With this respect, in the same figure we report also the glass transition temperatures T_G of aqueous solutions of glycerol (Rasmussen and MacKenzie, 1971), which decrease ~35 K in the investigated h region, similarly to T_d. Such a behavior suggests that the environment around the protein largely affects the dynamical processes that underlie the dynamical transition. This is consistent with the recent neutron scattering investigation of Reat et al. (2000), which found that, on a timescale one order of magnitude faster than that of the present experiment, the general features of the protein dynamical transition depend on the solvent properties. The

glass transition temperature separates the viscoelastic state from the elastic glassy state (Angell, 1995). In glass-forming liquids T_G is usually smaller than T_C, the ratio T_C/T_G being ~1.15 for fragile compounds (Knaak et al., 1988) or somewhat higher for stronger systems (Brodin et al., 1996). Because, for pure glycerol $T_C/T_G \approx 1.24$ (Franösc et al., 1997), we have tentatively evaluated T_C for aqueous solutions of glycerol, within the hypothesis that the same relationship holds even for these systems. In addition, neutron scattering measurements have shown that the dynamical transition of pure glycerol is quite insensitive to differences on isotopic composition (Wuttke et al., 1995). Then, we may suppose that the estimated T_C trend will reasonably well represent the T_C of D_2O fully deuterated glycerol solutions, at least within a few Kelvin degrees. Fig. 6 shows that the curve representing T_C for aqueous solutions of glycerol fits quite well the dynamical transition temperatures of lysozyme embedded in the glycerol-water environment. However, the behavior of the solvated lysozyme T_d appears to be more complex than a simple monotone trend, especially in the low-hydration region from 0h to 0.3h. The dynamical transition temperature seems to be constant up to ~0.1h, whereas it drops quite abruptly at ~25–30 K at 0.2h. For higher water contents, T_d follows a slow decreasing trend toward a saturation value of ~195 K. This result indicates that the environment rules the protein dynamical transition on the whole, though some important details appear to derive from the inherent coupling of protein with the surrounding water and glycerol molecules. Within the framework of the double-well model, the present findings suggest that, as the water content in the mixtures increases, the energy barrier between the two states decreases rather markedly above 0.2h, thus allowing for a not negligible population probability of the excited state p_2 at lower temperatures. The skill of the environment molecules in supporting the anharmonic processes underlying the dynamical transition and, consequently, the internal protein flexibility would thus critically depend on the hydration degree. Rather surprisingly, the dynamical transition temperature of hydrated lysozyme powders (Pissis, 1992), which has been measured through thermally stimulated depolarization currents experiments, exhibits a qualitatively quite similar critical dependence on the water content. Also in this case, as shown in Fig. 7, T_d decreases mainly between 0.13h and 0.29h. A variety of techniques, such as electron spin resonance studies of spin label (Rupley et al., 1980), NMR (Rupley and Careri, 1991), Mossbauer spectroscopy (Belonogova et al., 1978), hydrogen isotope exchange (Poole and Finney, 1983), positron annihilation lifetime spectroscopy (Gregory and Chai, 1993), and Rayleigh scattering of Mossbauer radiation (Goldanskii and Krupyanskii, 1989), provide a considerable body of evidence that demonstrates that the internal dynamics of proteins recovers between 0.1h and 0.2h. This threshold hydration level corresponds to a condition where, after having completed the hydration of

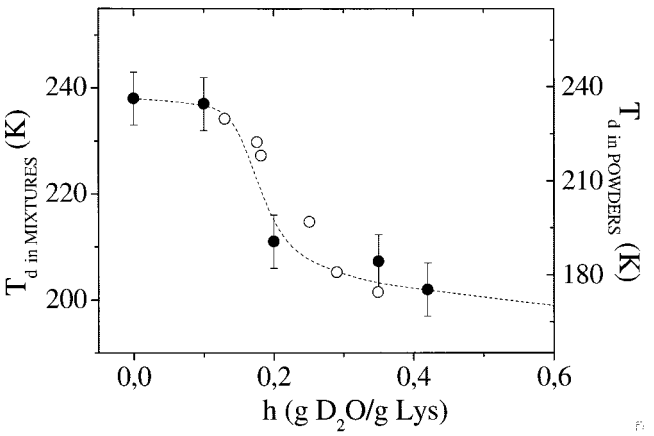

FIGURE 7 T_d of lysozyme in glycerol-water mixtures (●) and of lysozyme hydrated powders (○) versus h. The dashed line is a guide for the eye.

ionizable side chains, water molecules are progressively added to main-chain carbonyls and other polar surface groups (Yang and Rupley, 1979). We also note that at ~0.15h the onset of enzymatic activity occurs (Rupley et al., 1980). Lysozyme in glycerol-water mixtures seems to reproduce an analogous behavior. Indeed, also the amplitude of the total mean square displacements increases with h in a nontrivial fashion, as Fig. 8 shows. At room temperature, the atomic mean square displacements exhibit at first a constant trend at the lowest hydration levels, whereas an abrupt rise occurs between 0.1h and 0.2h, which is then followed by a less evident increase, probably toward an asymptotic value at high hydration degrees. A similar dependence on h is revealed for $T = 250$ K and, despite the large error bars, also at 200 K. The analogous features exhibited by lysozyme solvated with glycerol-water and by hydrated powders suggest that water carry out its specific

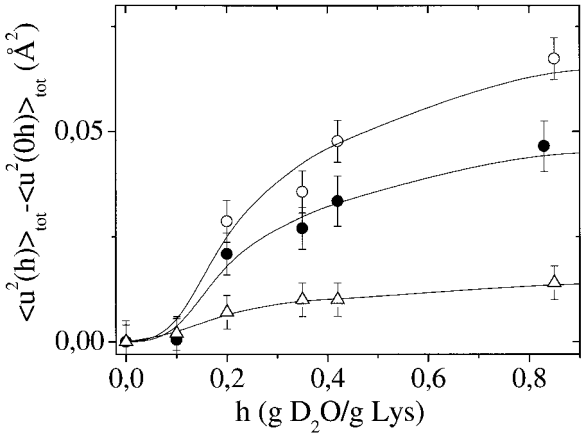

FIGURE 8 Increase of $\langle u^2 \rangle_{tot}$ as a function of h with respect to the anhydrous sample at 200 K (△), 250 K (●), and 300 K (○) versus h. The solid lines are guides for the eye.

plasticizing action in a similar way, in both the systems. At the low hydration degree $0.1h$, the protein mobility seems to be reduced as when it is put in the presence of only pure glycerol (Tsai et al., 2000, 2001), which acts as a stabilizer. However, for higher hydration levels, water begins to play its role of plasticizer, despite the adverse stabilizing action of the same glycerol. It may be speculated that this occurs through a mechanism of preferential hydration, as it happens for proteins in glycerol-water solutions (Gekko and Timasheff, 1981). The favorable interaction between glycerol and water, combined with the greater average affinity of the protein sites for water, promote the exclusion of glycerol molecules from the protein surface to minimize the free energy.

It has been recently proposed that the protein thermal stability is inversely correlated to the protein flexibility (Tang and Dill, 1998; Tsai et al., 2000, 2001). Thus, our results suggest that, by increasing the hydration level, the denaturation temperature should lower, especially above $0.1h$. Actually, this is what Bell et al. (1995) have observed. A deeper investigation on these topics is at the moment in progress.

CONCLUSIONS

We have studied how the global and, more in detail, the internal mobility of lysozyme are altered when the character of its environment, which is at first stabilizer in nature, is gradually shifted toward a plasticizer-like nature. We have thus seen that addition of water strongly affects the dynamical behavior of lysozyme solvated with pure glycerol. When the hydration degree increases, not only is the protein molecule able to globally diffuse, but also its internal dynamics appears to be more and more activated. In particular, both the onset and the amplitude of the hydrogen mean square displacements, which quantify on average the extent of the internal protein relaxations, depend in a nontrivial fashion on the hydration level. The protein internal dynamics seems to be ruled on the whole by the dynamical features of the environment. This is suggested by the similar dependence on h exhibited by T_d of lysozyme glycerol-water mixtures and T_G of glycerol aqueous solutions. However, the interplay between protein and surrounding glycerol-water molecules seems to be decisive to explain the marked decrease in the T_d trend above $\sim 0.1h$, just in correspondence to the sudden rise of the mean square displacements. Because the existence of a threshold value between $0.1h$ and $0.2h$ has been observed in many properties exhibited by simple hydrated protein powders, we may hypothesize that a preferential hydration effect takes place.

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