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ORIGINAL PAPER

Temperature dependence of protein dynamics as affected by sugars: a neutron scattering study

S. Magazù · G. Romeo · M. T. F. Telling

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Abstract Neutron scattering data on lysozyme–trehalose and lysozyme–sucrose aqueous mixtures, and on trehalose and sucrose aqueous mixtures are presented for a wide temperature range. Although the degree of protein coupling to solvent seems to be an open question in the literature, we present evidence that seems to be a firm link between a local dynamics of the protein with that of the glassy host. One of the objectives of this study was to explore the relationship between protein dynamics and glassy host. Measuring the $<u^2>$ of lysozyme mixtures, we arrive at a qualitative description of how their thermal stability is affected by the presence of two sugars at different temperatures. Whereas the Q dependence of the elastic incoherent structure factor gives information about the geometry and the amplitudes of the motions.

Introduction

The evidence for stabilizing effects of sugars has led to their routine usage in formulating biopharmaceuticals in order to improve their long-term storage and delivery (Pestrelski et al. 1993; Remmele et al. 1997; Shamblin et al. 1999; Hancock and Zografi 1997; Franks et al. 1991). The degree of change in thermal stability was dependent on the type of excipient. Our studies are in the framework of the relations structure–dynamics–function of biological macromolecules, and deal with the temperature dependent transition in the dynamic behaviour of proteins, with cessation of anharmonic motions below this temperature. It is generally accepted that proteins require internal flexibility for their activity. The movements required are relatively poorly characterised. Of particular interest are the role in activity of the fast (i.e., picosecond time-scale) fluctuations and to what extent they are coupled to the structural changes, which may take place on time-scales several orders of magnitude slower. Some protein functions have been observed to cease with the loss of equilibrium anharmonic dynamics as the proteins are cooled through the dynamic transition (Ferrand et al. 1993; Rasmussen et al. 1992; Doster et al. 1989).

Incoherent neutron scattering experiments provide information on the motion of the sample hydrogen atoms in a specific time and space window, given by the energy resolution and scattering vector modulus, Q, range. Because the scattering is "incoherent", the sample need not be crystalline or even monodisperse.

The glassy mixture, which shows suppressed local dynamics also show enhanced protein stabilization. Cordone (1999) found that trehalose suppressed the mean square displacement, msd, $<u^2>$, in myoglobin significantly in comparison to an aqueous environment. This led them and others (Zaccai 2000) to speculate that an effective lyophilization medium will suppress fast dynamics of the protein reflected in $<u^2>$. The implication that a suppression of local motions $<u^2>$ of a glassy host also indicate a suppression of similar motions of a protein located in that host assumes that the fast, local dynamics of the protein

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S. Magazù · G. Romeo (✉)
Physics Department and INFM,
Messina University, Messina 98166, Italy
e-mail: gio.romeo@tiscali.it

M. T. F. Telling
ISIS Facility, Rutherford Appleton Laboratory,
Chilton, Oxon OX11 0QX, UK

and the host are coupled. The degree of protein coupling to solvent seems to be an open question in the literature (Halle and Denisov 1995; Tarek and Tobias 2000; Bizarri and Cannistraro 2002; Sokolov et al. 2001). The Kramers' model (Kramers 1940) is frequently the starting point for considering a coupling between solvent and solute dynamics. A large body of work on protein dynamics in various solvents indicates that Kramers' theory does not always predict protein dynamics properly. Finally, we can assume that the notion of a persistent relation between fast dynamics of solute and solvent is consistent with our data. Such studies would be useful as they might further elucidate the important factors in the biopreservation efficacy of these glasses.

One approach to the study of protein dynamics is to increase the solvent viscosity and monitor both the alteration in dynamics and the impact on protein function. In this study lysozyme is embedded in a glassy matrix at several temperature. This matrix provides a means of both trapping the equilibrium population and reducing or eliminating large-scale protein motions. The introduction of sugars into the solvent medium has been found to stabilize biological macromolecules in solution (Shelby et al. 1972; Frigon and Lee 1972; Lee and Timasheff 1981). The basic observations are that these additives prevent the loss of enzymic activities (Frigon and Lee 1972), inhibit irreversible aggregation (Frigon and Lee 1972) or increase the thermal transition temperature of macromolecules (Neucere and St Angleo 1972). Trehalose is a diglucose sugar that confers to certain plant and animal cells the ability to survive dehydration (Leslie et al. 1995). Solutions of trehalose are form glasses when dried under appropriate conditions at biological temperatures (Green and Angell 1989). There is current interest in using trehalose as a means of preserving proteins and peptides at ambient temperature without the need for lyophilization (Carpenter et al. 1987). Then interactions between proteins and solvent components have been investigated for the sucrose/water system. We were particularly drawn to this question by our finding that high concentrations of sucrose stabilize brain tubulin in solution (Frigon and Lee 1972). The stabilizing effect of these additives has been attributed in the past to a lessened hydrogen bond-rupturing capacity of the medium (Gerlsma 1968) or to an induced change in topography (Hinton et al. 1969). The solvent and protein coupling dynamics are an interesting issue that is currently debated in the literature. In this picture, it is interesting to study the molecular mechanism of the solvent motion that is required to instigate the protein structural relaxation above a transition temperature (our work) or a critical hydration level (Cornicchi et al. 2006). Until the critical hydration level is reached, proteins do not function, and this level is analogous to a similar lack of protein function observed for temperatures below a dynamical temperature range of 180–220 K. Thus, we focus on a comparison of how glassy matrix in sucrose/water/protein sample and in the respective trehalose-enriched glass inhibits some protein dynamics.

Theory

Neutrons with a 1 Å wavelength and an energy close to 1 kcal/mol represent excellent probe to characterize thermal molecular motions and conformational changes in biological systems (Doster et al. 1989; Zaccai 2000; Smith 1991). In complex biological structures hydrogens move together with larger chemical groups and therefore their motion corresponds to the global thermal behaviour of the system (Bicout and Zaccai 2001; Reat et al. 2000). The intensity of the scattered neutrons splits into two contributions: the coherent and incoherent scattering. In the case of the hydrogenated solutions, the coherent contribution can be neglected entirely. Incoherent neutron scattering (INS) is a particularly useful tool for determining the hydration-dependent dynamics of proteins. INS probes motions on length scales of the order of Å and timescales ranging from femtoseconds to nanoseconds (Cornicchi et al. 2006). In the incoherent approximation, the dynamical structure factor is generally expressed as (Orecchini et al. 2002):

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

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 possible initial positions. The second term is the quasielastic 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.

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 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 $\tau_i = \hbar/\Gamma$. 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 $g(\omega)n(\omega,T)/\omega$ were $g(\omega)$ is the protein density of vibrational states and $n(\omega, T) = \left(e^{\hbar\omega/k_B T} - 1\right)^{-1}$ is the Bose factor (Lovesey 1988). Finally due to the finite resolution of the spectrometer, Eq. 1 must be convoluted with the experimental resolution function $R(Q, \omega)$. The so-called Debey–Waller factor in front of Eq. 1 describes the Gaussian Q decreasing due to the vibrational atomic msd $<u^2>$ (Paciaroni et al. 2002).

Experimental set-up

Aqueous solutions of ultrapure α,α-trehalose and sucrose, purchased by Aldrich-Chemie, were investigated at a molar fraction $\phi \sim 0.035$, $\phi = n_d/(n_d + n_w)$, n_d and n_w being the solute and water mole numbers, respectively. The solutions were prepared by using double distilled and deionized water. Lysozyme from hen egg white (Sigma, St Louis, MO) was used without further purification to prepare the two samples: lysozyme–trehalose–water and lysozyme–sucrose–water mixtures. Lysozyme powder (300 mg) was dissolved in 2.5 ml of H_2O and in the same volume of a solution containing 12% of trehalose and sucrose. Each suspension was deposited on a surface of aluminium plates and subsequently dried under controlled conditions, by exposing it to silica gel to achieve an hydration of ~0.22 (g solvent/g protein). The water content was monitored by measuring the increase in weight of the protein samples. Inelastic neutron scattering experiments were carried out at ISIS pulsed neutron facility (Rutherford Appleton Laboratory, Chilton, UK) using OSIRIS neutron instrument (Telling and Andersen 2005) in the PG002 configuration. In these conditions, the mean energy resolution was $\Gamma = 24.5$ μeV of full width at half-maximum, as determined by reference to a standard vanadium plate. Such energy resolution allows one to observe motions faster than approximately $2\pi\hbar/\Gamma \cong 170$ ps. The sample was contained in an aluminium cell with thicknesses 0.2 mm. Measurements were performed on trehalose/lysozyme aqueous solution at different temperatures between 150 and 250 K and, for comparison, sucrose/lysozyme aqueous solution in the temperature range: 150 K < T < 310 K.

Results and discussion

By the direct numerical integration of experimental data, we deduced the Debey–Waller factor. This factor provides direct information concerning the vibrational dynamics of the protein. For each temperature, the slope of the line gives the value of $<u^2>/3$. We plot the obtained values of $<u^2>$ as a function of absolute temperature (Fig. 1). $<u^2>$, the msd of the vibrational motion, as expected, increases with increasing temperature, reflecting increased thermal motion. As will be shown, the change in $<u^2>$ as a function of temperature may be used to predict the thermal stability of the protein. The harmonic region, where a mean environmental force constant can be calculated from the slope of the line (Zaccai 2000), is shown in the Fig. 1. The deviation from the straight line is called a dynamical transition (that begin at the dynamical transition temperature, $T_D \sim 220$ K), and this region corresponds to the anharmonic regime. This portion of the scan describes the physiological dynamics of the protein. The value of T_D and the change in $<u^2>$ as a function of temperature may be used to characterized the heat lability of a protein sample. A higher T_D suggest the protein is less susceptible to thermally induced unfolding. The sample with the smallest response to temperature change may be considered to be the most dynamically stable. In this picture, the lysozyme–trehalose aqueous mixture is more strong than the lysozyme–sucrose aqueous mixture which is reminiscent of the strong/fragile classification of glass forming liquids (Angell 1995). Fragile materials characterized by high density of configurational states are more susceptible to thermally induced structural changes during heating than strong materials, which have a low density of configurational states.

Below T_D, disaccharide appears to help proteins avoid denaturation by surrounding them by a continuous vitreous layer. This suggested an explanation in terms of dynamics for the protective effect of the sugar. The protein atoms are trapped within individual conformational substates,

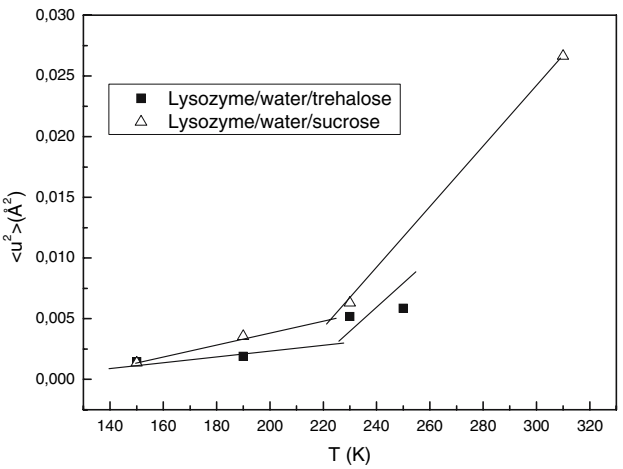

Fig. 1 Mean square displacement versus T for all the investigated samples

precluding the anharmonic motions that ultimate lead to unfolding. $<u^2>$ shows for lysozyme–sucrose–water sample a linear increase with temperature, typical of a harmonic behavior, for temperatures below the dynamic transition temperature, T_D. A strong increase of $<u^2>$ above this transition is also observed because of the onset of diffusive motions. There is a marked increase in mean fluctuation amplitudes with rise in temperature, showing that the components of the protein are becoming progressively more flexible. We see in Fig. 1 that $<u^2>$ evolves linearly with T below T_D for all samples, as is characteristic for a harmonic solid. Within this framework, the equipartition theorem is used to calculate an effective spring constant k. Stiffer harmonic vibrations have a larger k or a smaller temperature dependence of $<u^2>$. The harmonic oscillator assumption implicit in the determination of k is of course not appropriate above T_D. A force constant, in the strict sense is not defined in the case anharmonic environment at a temperature T can be quantified by a pseudo-force constant, k', calculated from the slope of the scan above the dynamical transition. However, as suggested by Zaccai (2000), it is still useful to think of reduced $<u^2>$ at this higher temperatures qualitatively as an increased environmental spring constant of the soft medium. A mean environmental force constant we can estimate for the samples from the temperature variation of the fluctuations and the comparison indicates that we have a larger K value for lysozyme–trehalose–water sample than for lysozyme–sucrose–water sample.

An important parameter that, usually, provides information on the geometry of the observed motion is the EISF determined experimentally as the ratio of the elastic peak intensity over the total signal. The presence of an EISF indicates that the observed motions have a localised diffusive nature. Figures 2 and 3 show the experimental $A_0'(Q)$ for each samples at different temperature. The internal motions of a protein take place over a large time scale, and some are not detected at experimental resolutions. Scattering by these 'immobile' protons makes a constant contribution to the pseudo-EISF $A_0'(Q)$. To represent this effect, we introduced the parameter p, which remains constant with changes in Q, to represent the fraction of protons in the protein that are considered to be immobile. Accordingly, A_0 can be written as $A_0(Q) = p + (1-p)A_0'(Q)$ where $A_0'(Q)$ is the pseudo-EISF of the hydrogen atoms, involved in detectable internal dynamics. It is possible identify a definite geometric shape from such a quantity, because these value have been fitted by the free diffusion in a sphere model (Volino and Dianoux 1980) and the fit give information about the radius. The fraction of immobile scatterers, p, is shown in Figs. 4 and 5 as a function of temperature. We found that p decrease as temperature increase. The temperature dependence of p before the

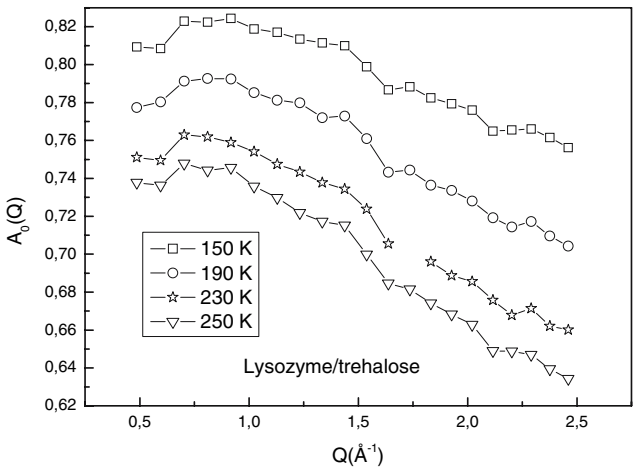

Fig. 2 Elastic incoherent structure factor, EISF, versus Q for the lysozyme–trehalose–water system

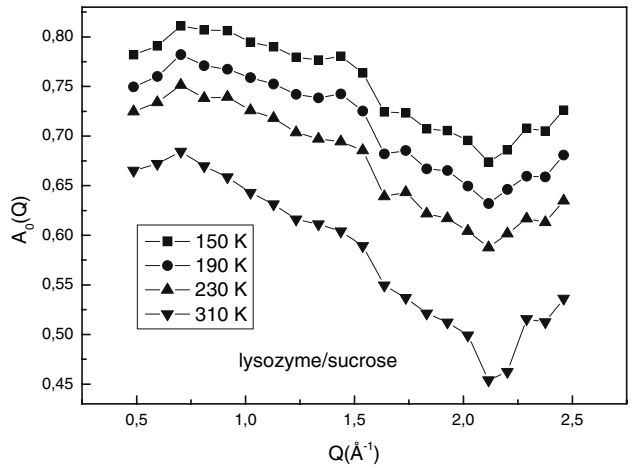

Fig. 3 Elastic incoherent structure factor, EISF, versus Q for the lysozyme–sucrose–water system

transition temperature can be explained if we consider that, at room temperature, the dominant contribution to internal motion is that of side chains exposed to the solvent. These side chains are free to move and to explore a large space. As temperature increases, the buried protons became more mobile. The observed decrease in the proportion p of immobile hydrogens is thus logical consequence.

Faster motions are taken into account by the Debey–Waller factor where $<u^2>$ gives the global average mean square displacements of vibrational motions of the hydrogens. Whereas the Q dependence of the elastic incoherent structure factor A_0' (Figs. 4, 5) gives information about the geometry and the amplitudes of the motions. For a more detailed analysis we could applied the "diffusion inside a sphere" model introduced by Volino and Dianoux (1980). Here A_0 is given by $A_0'(Q) = \left[3\frac{\sin(Qr)-(Qr)\cos(Qr)}{(Qr)^3}\right]^2$ and is parametrized by the radius of the sphere r. This function

equals unity at $Q = 0$ and has a null value at $Q \approx 4/r$. A three-sites jump model would tend to $A_0'(Q) = \frac{1}{3}\left[1 + 2\frac{\sin(Qr\sqrt{3})}{Qr\sqrt{3}}\right]$, and r is the radius of the circle containing the three sites. In this case, there is a finite marked minimum at $Q \approx 2.6/r$. A different type of motion would give a different variation of A_0' with Q. Thus, in principle, by analyzing $A_0'(Q)$, we should be able to distinguish between several types of motion. It is noteworthy, that in the case of biological macromolecules the radius value r must be understood as averaged parameters, because the dynamic behaviour of the hydrogens in the protein is much more complex and can only be approximated by the model used here. In a liquid the rotational motion should be spherical, i.e., after a sufficiently long time, the protons have occupied all the points on a sphere, with equal probability and we use the model "diffusion inside a sphere" in this frame. Attempting to extract a radius of gyration from protein–trehalose–water and protein–sucrose–water data, drawn Fig. 6a, we obtain a size that is too small to be consistent with the radius of the hydrated disaccharides: about 6 Å as obtained from photon correlation spectroscopy measurement (Branca et al. 2003). The two models fit each experimental set of data with the same fit quality, but for clarity, the fitting curves are shown for each sample in Figs. 4 and 5 only for the three-sites jump model. The fit in Fig. 6b is good enough to account for typical distances expected for methyl hydrogen flips, for which $r = 0.99$ Å, or for diffusive liquid-like motions of surface side chains. Protons located in methyl groups tend to perform uniaxial rotational diffusion (e.g., three-site jump diffusion (Bee et al. 1985; Chahid et al. 1994). NMR analysis of methyl group rotational motion in lysozyme revealed this kind of motion as the major part of internal motion occurring on the picosecond time scale (Shirley and Bryant 1982). Although the two models produce the same fitting curves the parameter r is systematically higher in the three-site jump model than in the model of free diffusion in a sphere. It is not surprising because r does not represent the same type of radius in each of the two models (Fig. 6). Thus, we may think of the actual dynamics as a combination of them, the free diffusion model being more adequate at low values of r, and the site-jump at high values of r. In the previous works (Branca et al. 2001a, b; Magazù et al. 2001), it has been shown that, among disaccharides, trehalose has the greatest destructuring effect on the tetrahedral hydrogen bonded network of water. As a result, the amount of freezable water is reduced and the crystallization process obstructed. The different rigidity of both trehalose–water and sucrose–water solutions has been also characterized by means of elastic neutron scattering measurements (Magazù et al. 2005), providing information on the low-frequency dynamics and on the thermal msd through the temperature behaviour of the Debye–Waller factor. According to this study, the trehalose aqueous system plays the role, in comparison with sucrose aqueous solution, of the strongest system. Finally, it is interesting to compare the plot of $1/\langle u^2\rangle$ versus $1000/T$ (Figs. 7, 8) for the protein–glassy mixture data (OSIRIS measuremets, this

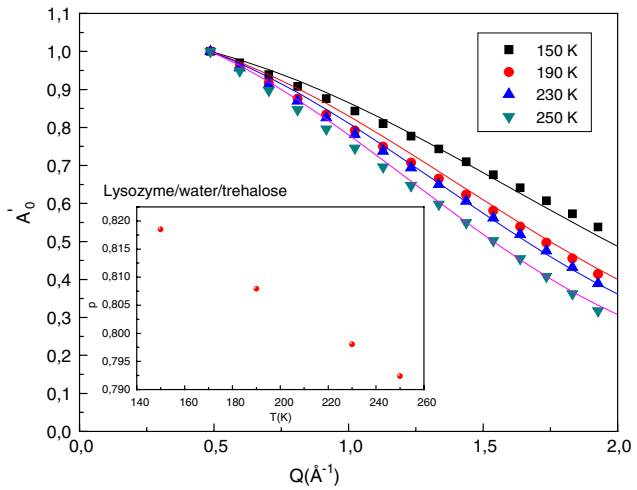

Fig. 4 $A_0'(Q)$ is the pseudo-EISF of the hydrogen atoms where the fitting lines result from the three-site jump model with the fraction of immobile scatterers, p, versus T for the lysozyme–trehalose–water system shown in the *inset*

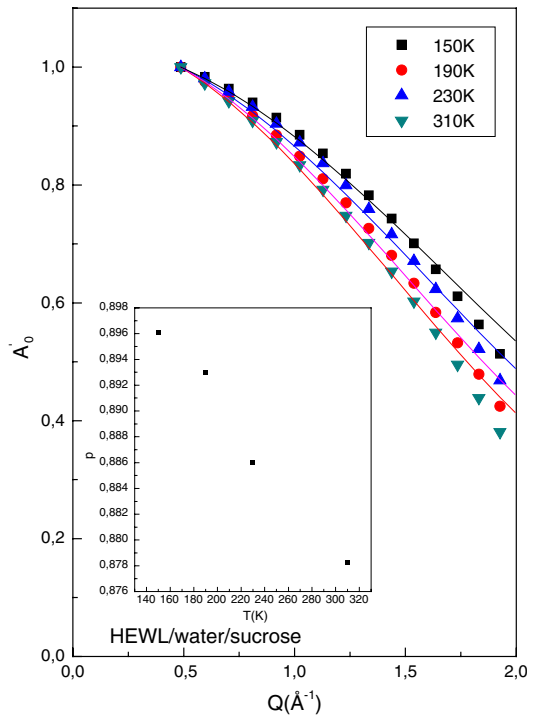

Fig. 5 $A_0'(Q)$ is the pseudo-EISF of the hydrogen atoms, where the fitting lines result from the three-site jump model with the fraction of immobile scatterers, p, versus T for the lysozyme–sucrose–water system shown in the *inset*

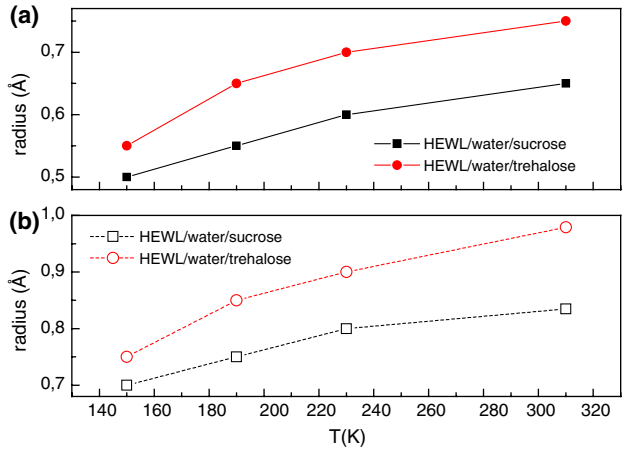

Fig. 6 Radius extract from pseudo-EISF of the hydrogen atoms versus T for all the investigated samples (hen egg white lysozyme, HEWL/water/disaccharide) by model of the free diffusion in a sphere (**a**) and three-site jump model (**b**)

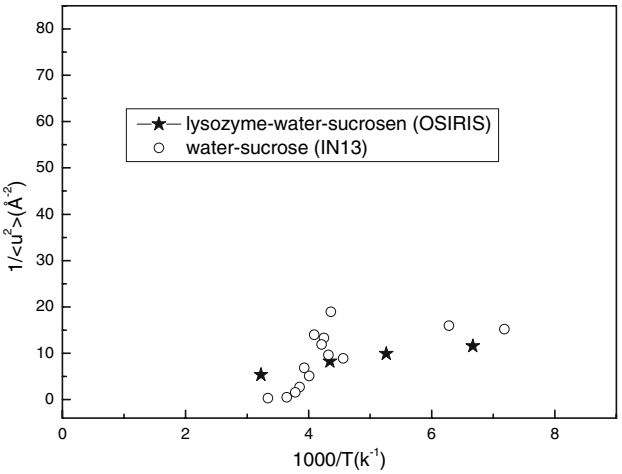

Fig. 8 Arrhenius plot $1/\langle u^2\rangle$ versus $1000/T$ for the lysozyme–sucrose–water system (OSIRIS measuremets, this work) and sucrose–water system (IN13 instrument, Magazù et al. 2005)

work) with that for the glasses data without protein obtained by previous neutron scattering findings (IN13 instrument, Magazù et al. 2005). The motivation for comparing $1/\langle u^2\rangle$ with Arrhenius plot comes from the expectation, based on theoretical arguments, that the timescale of local relaxations of the glass should scale exponentially with $1/\langle u^2\rangle$ (Hall and Wolynes 1987). The similarity between the IN13 and OSIRIS data are striking and it seems to be a firm link between the local dynamics of the protein with that of the host.

Concluding remarks

The neutron scattering method is able to separate flexibility, fluctuation amplitudes, and rigidity, a mean environmental force constant. This suggests that macromolecular thermoadaptation was achieved by evolution through selection of appropriate protein resilience. It should allow to refine our understanding of the relation between molecular dynamics and biological activity. The data show a cross-over in molecular fluctuations between harmonic and anharmonic dynamical regimes which permits the system to access multiple minima of its configuration space. While, an estimation of the EISF can give semi-quantitative information on molecular mechanism of he solvent motion that is required to instigate the protein structural relaxation above a transition temperature. The Q dependence of the structure factors, as measured for both samples at different temperatures, yields that increasing temperature A_0 decreases more strongly with Q, i.e., more quasielastic intensity at high Q values. This result was quantified by fitting the data with the diffusion inside a sphere model. In terms of this model we obtain for the protein/water/trehalose larger radii, and therefore a larger part of the conformational space explored by confined motions, with increasing temperature. Due to lower energy barriers between adjacent conformational substates a certain increase in temperature results in more pronounced thermal fluctuations as compared to the folded state. Despite the use of two different models for the hydrogen motions, free diffusion in a sphere and three-site jump, we obtain the same results from the fitting of the experimental data. This suggests that the conclusion proposed here are model independent.

Moreover, we show data collected by IN13 and OSIRIS instruments on sugar–water and sugar–water–protein mixture, respectively, and the similarities between the protein–sugar–water and sugar–water data are striking. Then, we assume that the dynamics of the protein and the host are coupled.

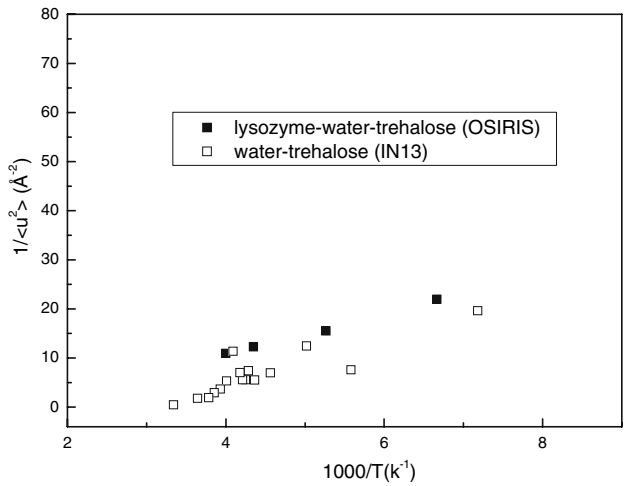

Fig. 7 Arrhenius plot $1/\langle u^2\rangle$ versus $1000/T$ for the lysozyme–trehalose–water system (OSIRIS measuremets, this work) and trehalose–water system (IN13 instrument, Magazù et al. 2005)

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