Eur Biophys J (2012) 41:361–367
DOI 10.1007/s00249-011-0760-x

ORIGINAL PAPER

EBSA
Biophysics in Europe

Bio-protective effects of homologous disaccharides on biological macromolecules

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Received: 1 July 2011 / Revised: 8 October 2011 / Accepted: 11 October 2011 / Published online: 30 October 2011

Abstract In this contribution the effects of the homologous disaccharides trehalose and sucrose on both water and hydrated lysozyme dynamics are considered by determining the mean square displacement (MSD) from elastic incoherent neutron scattering (EINS) experiments. The self-distribution function (SDF) procedure is applied to the data collected, by use of IN13 and IN10 spectrometers (Institute Laue Langevin, France), on trehalose and sucrose aqueous mixtures (at a concentration corresponding to 19 water molecules per disaccharide molecule), and on dry and hydrated (H_2O and D_2O) lysozyme also in the presence of the disaccharides. As a result, above the glass transition temperature of water, the MSD of the water–trehalose system is lower than that of the water–sucrose system. This result suggests that the hydrogen-bond network of the water–trehalose system is stronger than that of the water–sucrose system. Furthermore, by taking into account instrumental resolution effects it was found that the system relaxation time of the water–trehalose system is longer than that of the water–sucrose system, and the system relaxation time of the protein in a hydrated environment in the presence of disaccharides increases sensitively. These results explain the higher bioprotectant effectiveness of trehalose. Finally, the partial MSDs of sucrose/water and trehalose/water have been evaluated. It clearly emerges from the analysis that these are almost equivalent in the low-Q domain (0–1.7 Å^{-1}) but differ substantially in the high-Q range (1.7–4 Å^{-1}). These findings reveal that the lower structural sensitivity of trehalose to thermal changes is connected with the local spatial scale.

Keywords Bioprotection · Biological molecules · Homologous disaccharides · Lysozyme · Water · Elastic incoherent neutron scattering

Introduction

Hydrated disaccharides are nowadays the object of intense research efforts motivated both by fundamental research and by their biotechnological applications, e.g. in cosmetics and pharmaceuticals. In particular, among disaccharides, trehalose has received growing attention, because of both its wide role in nature and its potential use as a highly efficient natural bioprotectant. Trehalose and sucrose have the same chemical formula ($C_{12}H_{22}O_{11}$) (molecular weight $M_w = 342.3$), but different structures, which could account for their different effectiveness. More precisely sucrose (α-D-glucopyranosyl β-D-fructofuranoside) consists of a glucose ring (pyranose) in the α configuration and a fructose ring (furanose) in the β configuration; the α and β structures of the same monosaccharide differ only in the orientation of the OH groups at same carbon atom in the ring itself (mutarotation equilibria). Trehalose is a disaccharide of glucose (α-D-glucopyranosyl β-D-fructofuranoside)

Special Issue: Biophysics of cosmetics.

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consisting of two pyranose (six-membered) rings in the same α configuration, linked by a glycosidic bond between the chiral carbon atom C1 of the two rings. Both pure sugars form glasses at temperatures above ambient temperature, but the glass transition temperature (T_g) of sucrose is significantly lower than that of trehalose (350 K as opposed to 388 K for trehalose). Trehalose and sucrose aqueous mixtures have been characterized by light scattering, e.g. photon correlation spectroscopy and Raman scattering, by neutron scattering, and by simulation study, e.g. neutron diffraction, inelastic scattering QENS (Magazù 1996; Affouard et al. 2005; Magazù et al. 2006). These techniques furnish evidence that, compared with sucrose, trehalose has a higher solute–solvent interaction strength, a higher kosmotropic character, and greater capability to switch off molecular dynamics.

During recent years substantial efforts have been made in experimental, theoretical, and computational studies to clarify the microscopic nature of the dynamics of biological macromolecules. One phenomenon which has been widely debated, but not yet fully clarified, is the so-called *dynamic transition* in protein systems which, in the literature, is referred to as a sharp rise in the mean square displacement (MSD) of hydrated proteins relative to the dry sample, usually registered in the temperature range $T = 200$–240 K (Doster et al. 1989; Rasmussen et al. 1992; Zaccai 2000; Doster 2007; Sokolov et al. 2008). Basic understanding of the mechanism underlying the observed *dynamic transition* remains controversial and various models have been proposed: It had been ascribed to a sudden change in *effective elasticity* of the protein (Zaccai 2000), to motion of specific side groups (Lee and Wand 2001), to a specific fragile-to-strong crossover in the dynamics of hydration water (Chen et al. 2006), to the microscopic manifestation of the glass transition in the hydration shell (Doster et al. 2010), and to resolution effects because of a relaxation process within the experimentally accessible frequency window (Khodadadi et al. 2008).

It is well known that neutron scattering enables characterization of the structural and dynamic properties of a wide class of materials, for example polymers, glasses, and proteins. These properties can be described by the time-dependent spatial correlation function $G(r,t)$ introduced by Van Hove (1954), whose space–time Fourier transform corresponds to the scattering function $S(Q,\omega)$. When the system scattering cross section is mainly incoherent the relevant contribution to the time-dependent spatial correlation function is given by the self-distribution function $G_s(r,t)$, which is the probability of finding a given particle at a distance r from a given position after a time t (Van Hove 1954; Magazù 1996).

The experimentally obtained neutron scattering data also depend on the instrumental features of the spectrometer used. This implies that system observables, for example the distribution functions and the MSD, are affected by instrumental effects. In this regard, several contributions are reported in the literature (Becker and Smith 2003; Becker et al. 2004; Kneller and Calandrini 2007; Gabel and Bellissent-Funel 2007; Magazù et al. 2009, 2010a, b, 2011a). The experimentally accessible quantity in the *ω-space*, because of the finite energy instrumental resolution $\Delta\omega$, is the convolution of the scattering law $S(Q,\omega)$ with the instrumental resolution function $R(\omega;\Delta\omega)$, i.e. the *measured* scattering law $S_R(Q,\omega;\Delta\omega)$.

Recently (Magazù et al. 2011a), evaluation of the effects of the finite instrumental energy resolution has shown that when $\tau_{RES} > \tau$ the resolution effects are negligible whereas when $\tau_{RES} < \tau$ the resolution effects become important and the *measured* elastic scattering law becomes equal to the area under the resolution function; in this context it is important to stress that an inflexion point in the *measured* elastic scattering law occurs when the instrumental energy resolution of the spectrometer used intersects the inverse of the system relaxation time. It should be noted that such a circumstance is at the basis of a new experimental approach, i.e. resolution elastic neutron scattering (RENS); this approach, giving access to the elastic scattered intensity as a function of the instrumental energy resolution, enables characterization of the dynamic system of a wide class of complex materials (Magazù et al. 2011c) without using of any fitting procedure, which is an important asset for researchers using trans-disciplinary procedures. This finding suggests that, so far, transitions in the *measured* elastic scattering laws may have mistakenly been attributed to real transitions in the dynamic properties of the systems, instead of taking into account merely instrumental effects. More specifically, because a transition in the *measured* elastic scattering law as a function of temperature gives rise to a transition in the extracted MSD temperature behaviour, transitions in the MSDs may have been mistakenly attributed to transitions of the real dynamic properties of a given system. In this regard it has been shown that the so-called *dynamic transition* is an instrumental energy resolution effect, and more specifically, it appears when the characteristic system relaxation time intersects the resolution time (Magazù et al. 2011a).

Experimental

Experimental data were collected at the Institute Laue Langevin (Grenoble, France) using IN13 and IN10 spectrometers. These spectrometers are characterized by incident neutrons of relatively high energy (16 meV), enabling them to span quite a wide range of momentum transfer with two different energy resolutions.

More specifically, for the IN10 spectrometer the incident wavelength was 6.27 Å, the Q-range was 0.30–2.00 $Å^{-1}$, and the elastic energy resolution (FWHM) was 1 μeV, which corresponds to an elastic time resolution of 2,192 ps; for the IN13 spectrometer the incident wavelength was 2.23 Å, the Q-range was 0.28–4.27 $Å^{-1}$, and the elastic energy resolution (FWHM) was 8 μeV, which corresponds to an elastic time resolution of 274 ps.

Trehalose/19H_2O and sucrose/19H_2O hydrated powder systems, i.e. with the amount of water molecules corresponding to the first hydration shell of water at room temperature, and partially deuterated lysozyme in the dry state, in D_2O, and in H_2O, with and without disaccharides, at a hydration value of $h = 0.4$ (h = g of water/g of protein) were used.[1]

More specifically, we used disaccharides (nos T9531 and S9378; Sigma–Aldrich) and hen-egg white lysozyme protein (no. L6876; Fluka), and 99% pure D_2O liquid. The protein was dialyzed to remove salts and lyophilized. The lyophilized powder was hydrated at a hydration value of $h = 0.4$, and was used an ample time after mixing. Lyophilized lysozyme was directly used as the dry sample. More specifically, lysozyme in D_2O at a hydration value of $h = 0.4$, and lysozyme in disaccharide/H_2O at a hydration value of $h = 0.4$ (h = (g water + disaccharide)/(g protein)) were studied. This hydration value was chosen because the activity of proteins seems to be highly dependent on the presence of at least a minimum amount of solvent water (Gregory 1995; Careri 1998).

Data were collected by use of the two spectrometers in the temperature range 20–320 K. The empty cell contribution was subtracted and spectra were normalized to a vanadium standard. Data treatment from all three spectrometers was performed with *Lamp* software; other specific new software was also written and used for data analysis. More specifically, the SDF procedure (Magazù et al. 2009, 2010a, b, 2011b) was used for MSD evaluation.

[1] With regard to the water–disaccharide systems, the incoherent contribution is of 94%, of which, more specifically, 64% is related to the water and 36% to the disaccharide. This implies that the incoherent contribution of the water is predominant in the scattering data, which give information on the motion of water. As a result, the effects of the presence of the disaccharides on water dynamics can be studied.

With regard to the lysozyme/H_2O/sucrose sample, the incoherent contribution is of 92%, of which, more specifically, 61% is related to the protein, 30% to the water, and 9% to the sucrose. This implies that the incoherent contribution of the protein is predominant in the scattering data. Such circumstances, together with the fact that the dynamics of the protein and the dynamics of solvent are strongly coupled, as highlighted by Caliskan et al. (2004), indicates that in such cases we mainly obtain information about protein hydrogen motion whereas comparison of the lysozyme/D_2O and lysozyme/H_2O/sucrose systems gives information about the effects of the disaccharide on protein dynamics.

Result and discussion

In this work the effects of trehalose and sucrose on the dynamic properties of both water and hydrated lysozyme were studied by analysis of the EINS contribution. More specifically, the self-distribution function (SDF) procedure presented in previous work (Magazù et al. 2008, 2009, 2010a, b, 2011b) was applied. It is well known that such a procedure is essentially based on the determination of the SDF and on its use for evaluation of the average statistical values of the physical quantity of interest $\langle A \rangle$, in agreement with the statistical mechanics definition; in the specific case of the MSD evaluation, the dynamic observable A corresponds to the second power of the displacement, $\mathbf{r}^2$:

$$\langle \mathbf{r}^2 \rangle = \int_{-\infty}^{\infty} \mathbf{r}^2 G^{\text{self}}(\mathbf{r}) d\mathbf{r} \tag{1}$$

in which the spatial self-distribution function, which furnishes a probability density, can be normalized to the units:

$$\int_{-\infty}^{\infty} G^{\text{self}}(\mathbf{r}) d\mathbf{r} = 1 \tag{2}$$

In the case in which the system can be considered isotropic, the volume integral becomes dependent only on the scalar r:

$$\langle \mathbf{r}^2 \rangle = \int_{-\infty}^{\infty} r^2 \left[4\pi r^2 G^{\text{self}}(r) \right] dr \tag{3}$$

The SDF can be applied directly to the experimental data and to whichever function able to reproduce their behaviour; it represents an integral procedure which takes into account the global Q behaviour and in so doing enables reduction of the error in the $Q \rightarrow 0$ extrapolation.

In previous work (Meinhold et al. 2008; Magazù et al. 2009, 2010a; Kneller and Hinsen 2009) it has been shown that the intermediate incoherent scattering function can be written as a sum of Gaussian functions in which the single Gaussian is related to a specific single motion. As a result, the total intermediate scattering function does not have Gaussian behaviour, because a sum of Gaussians is not a Gaussian function. In particular, it is interesting to observe that, as Tokuhisa et al. (2007) showed, the dominant non-Gaussian contribution in the intermediate scattering law of a protein is because of the dynamic heterogeneity of the scatterers and that non-Gaussian effects on the single-particle level are small.

Because the Gaussian is an eigenfunction of the Fourier transform operator, starting from a sum of Gaussian functions for the intermediate incoherent scattering function,

Table 1 Scattering function normalization procedures and the resulting MSDs

Function	Transformation	Resulting function	MSD
S_R	Normalization	nS_R	$\langle r^2\rangle_R \rightarrow \langle r^2\rangle_R$ (correct)
S_R	Translation	$S_R + n$	$\langle r^2\rangle_R \rightarrow \langle r^2\rangle_R/1 + n$ (incorrect)
$Ln(S_R)$	Normalization	$nLn(S_R)$	$\langle r^2\rangle_R \rightarrow n\langle r^2\rangle_R$ (incorrect)
$Ln(S_R)$	Translation	$Ln(S_R) + n$	$\langle r^2\rangle_R \rightarrow \langle r^2\rangle_R$ (correct)
S_R	Lowest temperature normalization	$S_R(T)/S_R(T_0)$	$\langle r^2\rangle_R(T) \rightarrow \langle r^2\rangle_R(T) - \langle r^2\rangle_R(T_0)$ (incorrect)

A transformation is considered correct when it does not modify the MSD. In contrast, a transformation is considered incorrect when the relative MSD depends on the transformation parameter. This analysis is based on the circumstance that the MSD is connected to the ratio of the neutrons scattered at one Q to the neutrons scattered at the other Qs

the SDF can be written as a sum of Gaussian functions, as reported elsewhere (Magazù et al. 2009; Magazù et al. 2010a; Magazù et al. 2010b):

$$G^{\text{self}}(r) = \sum_n A_n G_n^{\text{self}}(r) = \sum_n \frac{A_n}{16(\pi a_n)^{3/2}} \exp\left(-r^2/4a_n\right) \tag{4}$$

in which $\sum_n A_n = 1$.

In this case the MSD becomes:

$$\left\langle \mathbf{r}^2 \right\rangle = 6 \sum_n A_n a_n = \sum_n A_n \left\langle \mathbf{r}^2 \right\rangle_n \tag{5}$$

in which the partial SDFs and the partial MSDs are present. This formula emphasises that the MSD corresponds to a weighted sum of the different displacement contributions present in the system. It is important to stress the meaning of the partial MSDs—these may represent the MSDs associated with specific spatial observation windows and, as a consequence, are related to specific motion. Therefore this procedure enables one to obtain the autocorrelation function $G_{\text{self}}(r,t^*)$ versus r, together with its different partial contributions, and to determine the partial MSDs, their weights, and the total MSD.

Let us now consider the *measured* scattering law, which is a function of Q and represents the number of elastically diffused neutrons within a given the solid angle. Therefore a normalization consisting in a multiplicative factorization, i.e. $S_R \rightarrow nS_R$, is an allowed transformation able to rescale the data, because it does not change the proportionality relationship between the scattering intensity at different Q values. This transformation may be useful, for example, to give the same intensity value (e.g. at the origin) for different spectra collected at different temperatures and does not produce any change in the MSD evaluation.

In this regard, it should be taken into account that, in contrast, the same kind of normalization applied to the logarithm of the *measured* scattering law is a misleading procedure, because it leads to incorrect evaluation of the MSD value. In fact, the normalization of the logarithm of the *measured* scattering law corresponds to a power elevation of the *measured* scattering law, i.e. $nLn(S_R) \rightarrow (S_R)^n$, which furnishes an incorrect MSD, i.e. $n\langle r^2\rangle_R$.

On the other hand, as far as the logarithm of the *measured* scattering law is concerned, it can be observed that the proper transformation is the translation corresponding to normalization of the *measured* scattering law: $Ln(S_R) + Ln(n) \rightarrow nS_R$.

Finally, normalization of the *measured* scattering law obtained at a given temperature (usually the lowest) is again wrong, because it changes the relationship between the *measured* scattering law at different Q values; this procedure would cause a shift of the MSD, assigning it the value of zero at the lowest temperature: $S_R(T)/S_R(T_0) \rightarrow \text{MSD} = \langle r^2\rangle_R(T) - \langle r^2\rangle_R(T_0)$. In Table 1 the above normalization procedures are reported.

In this regard it is noticeable that the SDF procedure clarifies how correct normalization of both the scattering functions and the MSD trends should be performed.

By applying the SDF procedure to water–disaccharide systems the total and partial SDFs and the total and the partial MSDs were determined. In Fig. 1a the total MSDs of water–trehalose and water–sucrose systems are shown. It can be seen that above the glass transition temperature of water the MSD of water with trehalose is smaller than the MSD of water with sucrose; this circumstance suggests that the hydrogen bond network of the water–trehalose system is stronger than that of the water–sucrose system.

For spatial scale analysis, in Fig. 2a, b the partial SDFs as a function of r, at $T = 284$ K, for the water–sucrose and water–trehalose systems, respectively, are shown. It is interesting to observe that the low-r SDFs significantly differ from each other whereas the high-r SDFs are almost equal. Figure 2c, d shows the normalized SDF obtained and their partial contributions as a function of r, at $T = 284$ K, for the water–sucrose and water–trehalose systems, respectively. As can be seen, the different kinds of motion are spatially well separated within the accessible Q range; furthermore the SDF very closely follows the first partial SDF in the range 0–0.5 Å and the second one in the range 0.5–5.0 Å.

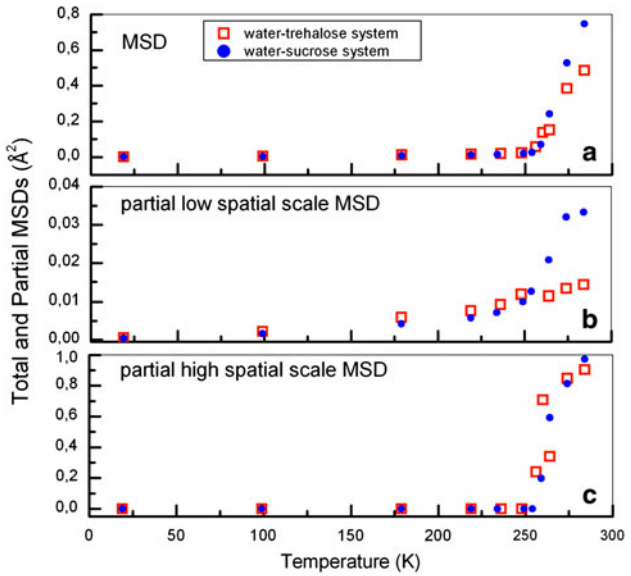

Fig. 1 **a** Total MSD, **b** partial MSD associated with *low-r* spatial domain, and **c** partial MSD associated with *high-r* spatial domain as a function of temperature for water–trehalose and water–sucrose systems in the temperature range 20–287 K obtained from data collected by the IN13. As can be seen in (**a**), above the glass transition temperature of water the MSD of water with trehalose is smaller than the MSD of water with sucrose; this explains the higher bioprotectant effect of trehalose compare with sucrose. Furthermore (**b**, **c**), the partial MSD behaviour is equivalent in the *high-r* domain whereas it is different in the *low-r* domain. This suggests that the highest structure sensitivity of sucrose should be a small spatial scale effect

Starting from these partial SDF functions, the partial contribution to the MSD can be evaluated. Figure 1b, c shows the partial MSDs for the water–sucrose and water–trehalose systems, evaluated by the SDF procedure, in the temperature range 20–287 K, in the *high-r* and *low-r* domains, respectively. As can be seen, the partial MSD behaviour of sucrose and trehalose are almost equivalent in the *high-r* domain, whereas they are noticeably different in the *low-r* domain. This finding suggests that the higher structural sensitivity to thermal changes of sucrose compared with trehalose is connected with the local spatial scale.

This finding is in agreement with inelastic neutron scattering results on relaxation versus vibration contribution analysis, i.e. the boson peak, indicating the presence of greater suppression of local fast dynamics in the trehalose–water system than in the sucrose–water system. In particular, such dynamic suppression correlates with weaker temperature dependence of viscosity and with lower fragility, which explains the higher bio-protectant effectiveness of trehalose compare with sucrose (Magazù et al. 2010c). Furthermore, this finding is in agreement with other experimental findings obtained by inelastic neutron scattering which reveal a higher downshift of the OH the intramolecular stretching contribution for the trehalose–water system, which indicates a stronger hydrogen bonded network in the trehalose–water system than in the sucrose–water system (Branca et al. 2003).

In the following text determination of the effects on hydrated protein dynamics of the presence of sucrose and trehalose will be discussed.

In Fig. 3 the MSDs as a function of temperature, in the range 20–287 K, obtained by the SDF procedure are shown for dry and hydrated lysozyme, with and without sucrose. As can be seen, the *dynamic transition* temperature is $T_D = 220$ K for the system without disaccharides, whereas the presence of disaccharides shifts the *dynamic transition* to higher temperature, at approximately $T = 255$ K.

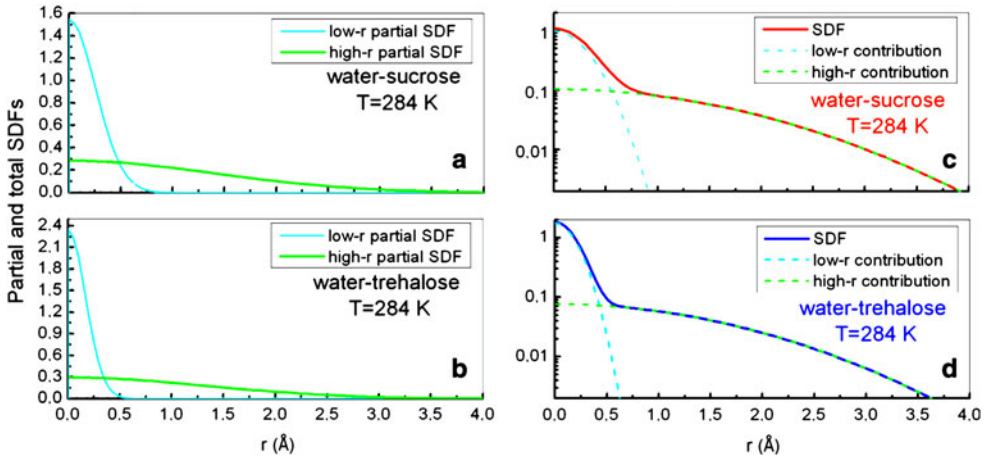

Fig. 2 Partial SDFs as a function of r at $T = 284$ K for **a** the water–sucrose system and **b** the water–trehalose system. As can be seen, the different kinds of motion are spatially well separated within the accessible Q range. In particular, the *high-r* partial SDFs are equal whereas the *low-r* partial SDFs differ from each other. Total normalized SDF as a function of r at $T = 284$ K for the water–sucrose and water–trehalose systems are shown in **c** and **d**, respectively, together with their partial contributions. The SDF very closely follows the first partial contribution in the range 0–0.5 Å and the second one in the range 0.5–5.0 Å

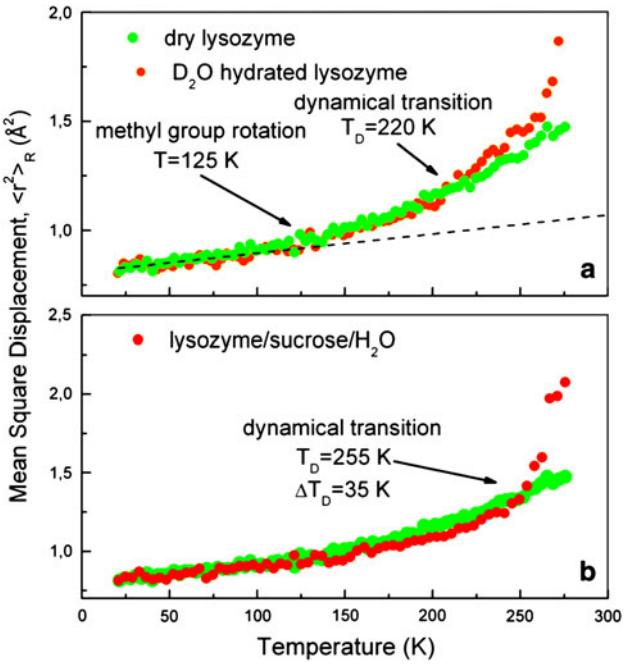

Fig. 3 MSDs of lysozyme. **a** Comparison between the MSD temperature behaviour of dry and D_2O-hydrated lysozyme, obtained from data collected by use of the IN10. The *dynamic transition* temperature is $T_D = 220$ K. **b** Comparison between the MSDs obtained for H_2O-hydrated lysozyme with sucrose and dry lysozyme by use of the IN10 spectrometer. The presence of disaccharide shifts the *dynamic transition* to a higher temperature value of $T = 255$ K

In this respect, it should be taken into account that recently the nature of the *dynamic transition* has been discussed in (Magazù et al. 2010a, 2011a), where it has been shown that the so-called *dynamic transition* is a finite instrumental energy resolution effect, and, more specifically, it appears when the characteristic system relaxation time intersects the resolution time, it does not imply any transition in the dynamic properties of systems. On this basis, as a result, considering the instrumental resolution effects in the EINS and the MSDs of Fig. 3, we can conclude that the system relaxation time of protein in a hydrated environment with disaccharides is longer than that without them, in other words the presence of the disaccharide slows the system dynamics. This result is in agreement with that reported in the literature and explains the bioprotectant effectiveness of trehalose and sucrose (Becker et al. 2004; Smith et al. 2004; Talon et al. 2004; Piazza et al. 2005; Lelong et al. 2007; Ciliberti et al. 2006; Di Fonzo et al. 2011; Bellavia et al. 2011).

Conclusion

In this work elastic incoherent neutron scattering was used to determine the effects of trehalose and sucrose on water and on hydrated lysozyme dynamics. In particular, the SDF procedure was applied to experimental data to obtain the MSD of the water–sucrose and water–trehalose systems with 19 H_2O molecules per disaccharide molecule, and on dry and hydrated (H_2O and D_2O) lysozyme with and without disaccharides. Furthermore, the normalization procedures used both on the EINS intensity profile and MSD are discussed. As a result, above the water glass transition temperature, the MSD of water–trehalose system is smaller than that of the water–sucrose system; this reveals that the hydrogen bond network of the water–trehalose system is stronger than that of the water–sucrose system. Furthermore, the partial MSDs of the water–sucrose and water–trehalose systems have also been evaluated. These are almost equivalent in the low-Q domain (0–1.7 $Å^{-1}$) whereas they differ substantially in the high-Q domain (1.7–4 $Å^{-1}$). This suggests that the higher structural sensitivity of sucrose is connected with the local spatial scale. These results explain the higher bioprotectant effectiveness of trehalose compared with sucrose.

Finally, considering the instrumental resolution effects in EINS, some dynamic information is discussed. In particular, the results obtained indicate that the system relaxation time of water with trehalose is longer than that with sucrose. Furthermore, with regard to the effect of disaccharides on protein dynamics, the experimental findings reveal that the system relaxation time of protein in a hydrated environment with disaccharides is longer than that without them. All these experimental results, which explain the high bioprotectant effectiveness of homologous disaccharides on protein system stability, with trehalose being more effective, have important applicative implications in both cosmetics and pharmaceuticals, in which, nowadays, one of the most topical subjects is the thermal and mechanical stability of high value-added products.

Acknowledgments The authors wish to acknowledge Le STUDIUM for strong support of this research. The Institut Laue-Langevin (ILL), Grenoble, France, is also acknowledged for beam time on the IN10 and IN13 spectrometers. A special acknowledgement is addressed to Dr C. Mondelli and Dr M.A. Gonzalez for precious discussions and guiding support during these experiments.

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