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# Protein dynamics by neutron scattering

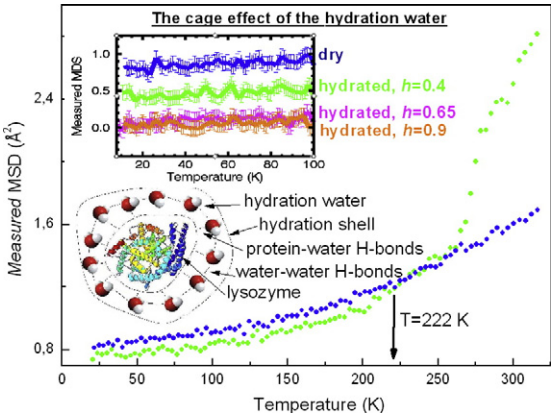
Antonio Benedetto *

*School of Physics, University College Dublin — UCD, Belfield Campus, Dublin 4, Ireland*
*School of Medical Sciences, Sydney Medical School, The University of Sydney, Anderson Stuart Building F13, Sydney, NSW 2006, Australia*

## HIGHLIGHTS

- An explicative hypothesis on the nature of the protein dynamical transition is formulated.
- A connection between the protein dynamical transition and the fragile-to-strong dynamical crossover is presented.
- A "cage-effect" resulting from the hydration water on the protein surface is observed and subsequently linked to the FSC.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Neutron scattering techniques represent a powerful tool for characterizing both the structure and dynamical properties of bio-systems, for example, proteins and membranes interacting with their solvents. In this paper, Elastic Neutron Scattering (ENS) data collected at the Institut Laue-Langevin (Grenoble, France) on dry and $D_2O$ hydrated lysozyme by varying hydration level are presented, and compared with previously published data on the same protein system, also with the addition of bio-protectants. The data have been collected with three different spectrometers, i.e. IN13, IN10 and IN4. This set of ENS data gives direct access to the temperature behavior of both (i) the Mean Square Displacement (MSD) and (ii) the characteristic system relaxation time. As a result, an explicative hypothesis on the relationship between the so-called "protein dynamical transition" (PDT) and the "fragile-to-strong dynamical crossover" (FSC) is formulated. Furthermore, by taking into proper account the effect of the finite instrumental energy resolution of the used spectrometers, the vibrational MSD of dry and hydrated lysozyme is calculated. The vibrational MSD of the lysozyme in the dry state resulted to be higher than the one in the hydrated state; the latter reaches the former at a temperature value of $T = 220$ K that corresponds to the temperature at which the FSC occurs. As a result, a cage effect resulting from the hydration water on the protein surface is hypothesized and subsequently linked to the FSC.



## 1. Introduction

During the last years considerable efforts have been addressed, through experimental, theoretical and computational studies, to clarify the microscopic structural and dynamical properties of biological

* Tel.: +353 1 716 1794.
*E-mail address:* antonio.benedetto@ucd.ie.

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macromolecules in different environments. In this paper, more specifically, the attention will be focused on two dynamical-observables that are "directly" accessible by neutron scattering techniques, i.e. the Mean Square Displacement (MSD), and the system characteristic relaxation time ($\tau$). These are usually measured as a function of temperature, and also by varying other physico-chemical parameters, for example, pressure, pH, concentration and hydration level. In particular, connected to these two dynamical observables, there are two different important and largely debated phenomena: the "protein dynamical transition" (PDT), and the "fragile-to-strong dynamical crossover" (FSC). The PDT is defined by a sharp rise in the temperature behavior of the MSD of hydrated proteins with respect to the dry ones, and it is usually registered in the temperature range T = 200–240 K [1–5]. On the other hand, the FSC is defined as a transition from an Arrhenius to a non-Arrhenius behavior in the temperature behavior of the characteristic relaxation time ($\tau$) of the hydrated protein systems, and it occurs around T = 220 K [6–10].

Both of these observables can be calculated by neutron scattering techniques (and can be also determined by computer simulations). More specifically, MSD is deduced from the *measured* neutron elastic scattering intensity $S_R(Q,\omega = 0,\Delta\omega)$, whereas the characteristic relaxation time is deduced from the *measured* neutron quasi-elastic scattering intensity $S_R(Q,\omega,\Delta\omega)$.

The interest in the PDT was stimulated by the fact that the measurable biochemical activity of proteins usually appears around the same temperature range [2,11]. For example, J. Pieper et al., in the case of hydrated Photosystem (PS) II, have demonstrated that the PDT occurs at the same temperature value at which the electron transport efficiency highly increases [12]; however, several exceptions exist [13–15]: e.g. in Ref. [15] the authors have shown that biological activity is also present in nearly dehydrated conditions. Furthermore, since the PDT is absent in dehydrated systems, it has been related to the dynamics of the hydration shell or at least coupled to it.

Various and different models have been proposed to explain the mechanisms underlying the observed PDT. Such a *transition* had been ascribed to a sudden change in an *effective elasticity* of the protein [3], to motions of specific side groups [11], to a specific fragile-to-strong crossover in dynamics of hydration water [6], to the microscopic manifestation of the glass transition in the hydration shell [16], and to a resolution effects due to a relaxation process that enters the experimentally accessible frequency window [17]. Furthermore, in Ref. [18] the authors proposed a model in which two different transitions take place at Tg and Td, and in Ref. [19] activation barriers and free energy values obtained for the PDT suggest to the authors to make a connection with the two-well interaction potential of supercooled-confined water.

On the other hand, the determination of the FSC, and its connection with the PDT have been made by Chen and coworkers. More specifically, performing different quasi-elastic neutron scattering (QENS) experiments, Chen and coworkers [6] have evaluated the temperature behavior of the mean characteristic time of the hydration water in hydrated lysozyme ($h$ = 0.3 g water/g protein); this latter shows a transition from an Arrhenius to a non-Arrhenius behavior at a temperature value of T = 220 K, which the authors put in correspondence with the PDT registered as a kink in the *measured* MSD at the same temperature value. This circumstance suggests to the authors that the kink represents a *time-scale independent* transition that really occurs in the system due to hydration water. Furthermore, with reference to confined water the authors have shown that the FSC takes place at T = 224 K at ambient pressure for confined water in silica nanopores [7,8], at T = 220 K in DNA [9] and at T = 220 K in RNA [10].

Furthermore, investigating the same system of Ref. [6] (with $h$ = 0.4) by both QENS and dielectric spectroscopy techniques, Sokolov and coworkers in Ref. [17,20,21] have not found that at T = 220 K any change in the system mean characteristic time, but a secondary fast process, related to water molecules, appears at temperatures lower than T = 220 K. The mean characteristic time of Chen et al. coincides with the main process for temperature values higher than T = 220 K and with the secondary fast relaxation for lower temperature values. Furthermore, Swenson et al. in Ref. [22,23] showed that in confined water the cooperative $\alpha$ relaxation vanishes at about T = 200 K implying that, above it, a merging of the $\alpha$ and $\beta$ relaxations takes places.

In the present paper, neutron experimental data (partially presented in ref. [40,42]) obtained on dry and hydrated lysozyme with and without the addition of two bio-protectants, i.e. trehalose and sucrose, are compared. In particular, for studying the effect of the hydration on the vibrational part of the MSD, new experimental data obtained at different hydration levels are presented here for the first time, i.e. dry, h = 0.4, h = 0.65 and h = 0.9. More specifically, neutron elastic spectra were collected with three different spectrometers working at three different instrumental energy resolutions, i.e. 200 μeV for IN4, 8 μeV for IN13 and 1 μeV for IN10. The idea of measuring the same systems with these different well-separated instrumental energy resolution values was stimulated by the fact that in this way should be possible to determine (i) how the PDT is affected by the energy resolution of the employed spectrometer, and, by using a novel protocol in data analysis, to (ii) shed a light on the connection between the PDF and the FSC.

As a result, starting from the connections between the used instrumental energy resolution with both the *measured* elastic scattering intensity and the extracted MSD (on this regard, several contributions are reported in literature during the last years [24–31]), it is shown that the observed PDT does not require necessarily any discontinuous change in the temperature behavior of the system relaxation time, and hence it is not necessarily connected to a real transition in the dynamical properties of the system. More specifically, the kink in the *measured* MSD temperature behavior appears when the system relaxation time crosses the instrumental resolution characteristic time.

Furthermore, by taking into correct account the effect of the finite instrumental energy resolution of the used spectrometers, the vibrational MSD of dry and hydrated lysozyme is calculated. The vibrational MSD of the lysozyme in the dry state resulted to be higher than the one in the hydrated state; the latter reaches the former at a temperature value of T = 220 K that corresponds to the temperature at which the FSC occurs. As a result, a cage effect resulting from the hydration water on the protein surface is considered and subsequently linked to the FSC.

## 2. Experimental section

Elastic Neutron Scattering (ENS) experiments were performed at the large neutron scale facility Institut Laue-Langevin (ILL, Grenoble, France) on dry and hydrated lysozyme samples by using three different spectrometers, i.e. IN13, IN10 and IN4. These spectrometers have been chosen due to the well-defined distance in their instrumental energy resolutions that give a well-separated accessible time windows, as previously described in ref. [40,41]. In particular: 200 μeV for IN4, 8 μeV for IN13 and 1 μeV for IN10.

The experimental set up for IN4 was: incident wavelength 3.60 Å; $Q$-range 0.3–4.5 $Å^{-1}$; elastic energy resolution (FWHM) 200 μeV, corresponding to a time of about 11 ps [32,33]. The experimental set up for IN13 was: incident wavelength 2.23 Å; $Q$-range 0.28–4.27 $Å^{-1}$; elastic energy resolution (FWHM) 8 μeV, corresponding to a time of about 270 ps [32,33]. The experimental set up for IN10 was: incident wavelength 6.27 Å; Q-range 0.30–2.00 $Å^{-1}$; elastic energy resolution (FWHM) 1 μeV, corresponding to a time of about 2190 ps [32,33]. The relationship between the elastic energy resolution and the corresponding resolution time is extensively reported in Ref. [32,33].

Hen-egg white lysozyme protein (no. L6876; Fluka), disaccharides (no T9531 and S9378; Sigma-Aldrich), and 99% pure $D_2O$ have been used. The protein was dialyzed to remove salts and lyophilized. The lyophilized powder was hydrated at hydration values of h = 0.4, h = 0.65 and h = 0.9, and was used an ample time after mixing. Lyophilized lysozyme was directly used as the dry sample.

More specifically, for studying the effect of hydration water and how this changes when bio-protectants are added, as previously described [40], lysozyme in $D_2O$ at a hydration value of h = 0.4, and lysozyme in sucrose/$H_2O$ at a hydration value of $h$ = 0.4, h = ($g$ of water + disaccharide) / ($g$ of protein) have been employed. It is believed, in fact, that 0.4 $g$ of water per $g$ of lysozyme is sufficient to cover the protein surface with a single layer of water molecules and to fully activate the protein functionality [34–36].

Whereas, for evaluating the effect of hydration level on the vibrational part of the MSD (i.e. the low-temperature MSD), a new set of hydration levels have been considered, i.e. dry, h = 0.4, h = 0.65 and h = 0.9, and presented here for the first time.

Measurements were performed for 12 h in the 20 K–320 K temperature range on dry and $D_2O$ hydrated lysozyme by IN10, IN13 and IN4 spectrometers, and on lysozyme/$H_2O$/sucrose and lysozyme/$H_2O$/trehalose mixtures by IN10. The analysis by varying the hydration level was performed by IN10. Raw data were corrected for empty cell and normalized by vanadium sample. Data treatment from all three spectrometers was performed with Lamp software; other specific new software was also written and used for data analysis.

## 3. Theoretical and numerical simulation background

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 [37], 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 $t$. The neutron scattering data depends also on the instrumental features of the spectrometer used. This implies that system observables, for example distribution functions and MSDs, are affected by instrumental effects. Due to the finite instrumental energy resolution, the experimentally accessible quantity is the measured scattering law, $S_R(Q,\omega,\Delta\omega)$, that is the convolution of the scattering law with the instrumental resolution function $R(\omega,\Delta\omega)$ [38,39]. In the simple case of elastic scattering, this corresponds to a time integral of the intermediate scattering function weighted in time by the instrumental resolution function:

$$S_R(Q,\omega=0;\Delta\omega)=S(Q,\omega)\otimes R(\omega;\Delta\omega)\Big|_{\omega=0}=\int_{-\infty}^{\infty}I(Q,t)R(t)e^{-i\omega t}dt\Big|_{\omega=0}=\int_{-\infty}^{\infty}I(Q,t)R(t)dt. \quad (1)$$

For properly understanding the meaning of the above time-integral, the numerical simulations and the temperature neutron measurements extensively presented in Ref. [40] are summarized in Fig. 1. Fig. 1 shows the comparison between the normalized time behavior of an intermediate scattering function $I(t;\tau)$ (black dashed line) at a fixed $\tau$ value ($\tau = 1.5 \cdot 10^3$ ps) and the resolution function $R(t;\tau_{RES})$ (in red) taken at three different $\tau_{RES}$ values ($\tau = 10^5$ ps for simulating the ideal case, $2\cdot10^3$ ps and $3\cdot10^2$ ps which correspond to the characteristic times of the energy resolution spectrometers IN10 and the IN13 respectively) together with the associated *measured* elastic scattering functions $S_R(\omega = 0,\Delta\omega)$ (in blue) obtained by Eq. (1). More specifically, in Fig. 1a it is shown that, due to the fact that $\tau \ll \tau_{RES}$, there is no any effect of the resolution function on the *measured* elastic scattering intensity, i.e. $S_R(Q,\omega = 0,\Delta\omega) = S(Q,\omega)$; in such a case the blue area that represents the $S_R(\omega = 0,\Delta\omega)$ coincides with the area under the intermediate scattering function, i.e. $S(\omega = 0)$. In Fig. 1b the case of the IN10 spectrometer is considered; it is shown how, in the *measured* elastic scattering intensity, the resolution function gives rise to a weighted

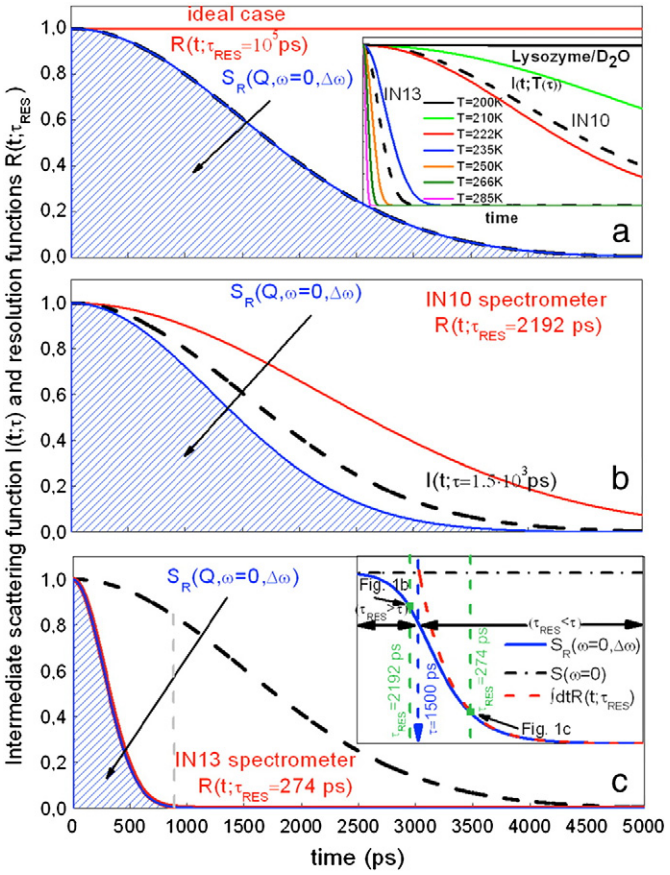


**Fig. 1.** Resolution effects in ENS (figures reproduced from ref. [32,40]). Comparison between I(t;$\tau$) at a fixed $\tau$ value (dashed black lines), R(t;$\tau_{RES}$) for different $\tau_{RES}$ values (continuous red lines) and the related $S_R(Q,\omega = 0,\Delta\omega)$ (the areas in blue). These latter, obtained considering Eq. (1), represent the output quantities of an ENS experiment on a system with a characteristic time $\tau$ in which $\tau_{RES}$ is the instrumental resolution time. (a) The resolution time is greater than the system characteristic time; there is no any resolution effect: the elastic measured scattering law and the elastic scattering law are coincident. (b) The resolution time becomes slightly smaller than the system characteristic time: the measured elastic scattering intensity is slightly smaller than the elastic scattering law (IN10 resolution function has been used). (c) The resolution time becomes much smaller than the system characteristic time. The resolution effect is evident: the measured elastic scattering law strongly differs from the elastic scattering law (IN13 resolution function has been used). (Inset — a) Temperature set of intermediate scattering function of lysozyme/$D_2O$ (data taken from Ref. [17]), and resolution functions of IN13 and IN10. The intermediate scattering functions cross the resolution function of IN10 at T = 220 K and of IN13 at T = 240 K. (Inset — b) *Measured* elastic scattering law, $S_R(Q,\omega = 0,\Delta\omega)$, as a function of energy resolution, $\Delta\omega = 1/\tau_{RES}$, for system with fixed $\tau$ value. Starting from the same intermediate scattering function used for the cases reported in this figure (for which the system characteristic time is $\tau = 1.5 \cdot 10^3$ ps), the $S_R(\omega = 0,\Delta\omega)$ has been evaluated as a function of $\Delta\omega$ according to Eq. (1); in particular, the shadowed areas of Fig. 1 have been calculated as a function of the instrumental energy resolution and the result is here reported (full curve); the vertical dashed arrow indicates the system relaxation time. The two cases in this figure are considered (squares) and two regimes are found: when $\tau_{RES} > \tau$ the resolution effects are negligible (case of Fig. 1b); when $\tau_{RES} < \tau$, the resolution effects become important (case of Fig. 1c) and the distance between the *measured* elastic scattering law and the elastic scattering law (horizontal dash-dotted line) increases. The change from one condition to the other one occurs when the system characteristic time intersects the resolution time; at the corresponding instrumental energy resolution value an inflection point occurs in the *measured* scattering law.

value of the intermediate scattering function; in this case the *measured* elastic scattering law $S_R(\omega = 0,\Delta\omega)$ (the blue area) is very close to the area under the intermediate scattering function (black dashed line) and the effects due to a finite resolution are quite small. In Fig. 1c the case of the IN13 spectrometer is taken into account; it clearly emerges that the resolution function originates from the exclusion of a portion of the intermediate scattering function (starting from the gray dashed line to longer times); in this case the *measured* elastic scattering law

$S_R(\omega = 0,\Delta\omega)$ (the blue area) is much smaller than the area under the intermediate scattering function.

The above-presented "theoretical" picture becomes "concrete" when the measurements are performed by varying physico-chemical parameters. In fact, for example, in a complementary way, by increasing temperature, the system relaxation time is gradually expected to decrease, giving rise to changes in the intermediate scattering function. In particular, in the inset of Fig. 1a, starting from the data collected by Sokolov et al. in Ref. [17] and reported in Fig. 2, the behavior of the main intermediate scattering function of $D_2O$ hydrated lysozyme at different temperature values are reported together with the resolution function of IN10 and IN13 (black dashed line). As it can be seen, by increasing the temperature the intermediate scattering function crosses at first the IN10 resolution function at about T = 220 K and then the IN13 resolution function at about T = 240 K. It crosses the IN4 resolution function at about T = 300 K.

The previous representation of the relationship between the intermediate scattering function, which describes the system properties, and the *measured* elastic scattering law, which is a mix of the system properties and of the instrumental features (see Eq. (1)), highlights the relationship between the system relaxation time and the instrumental resolution time. As a rule two regimes characterized by two distinct behaviors can occur. The first one deals with the case in which the resolution time is higher than the system relaxation time, i.e. $\tau_{RES} > \tau$ (see Fig. 1a and b), under this circumstance the resolution effect is quite small. The second one makes reference to the case in which the resolution time is shorter than the system relaxation time, i.e. $\tau_{RES} < \tau$ (see Fig. 1c), in this circumstance the resolution effect becomes relevant.

In order to better show what quantitatively happens, in the inset of Fig. 1c, the *measured* elastic scattering law $S_R(\omega = 0,\Delta\omega)$ as a function of the instrumental energy resolution, $\Delta\omega_{RES}$, is shown. In agreement with the latter, at the temperature value at which the resolution time intersects the system relaxation time, an inflection point occurs in the *measured* elastic scattering law. Under such circumstance, e.g. at the same temperature value, a kink occurs in the temperature behavior of the *measured* MSD. The meaning of this kink is that, at such a temperature value, the system relaxation time becomes equal to the characteristic time of the employed spectrometer.

More specifically, the evaluation of resolution effects on the *measured* scattering law can be analyzed by using the concept of the "equivalent time" $t^*$ by applying the theorem of integral average [41] to Eq. (1); as a result, a master curve for the equivalent time has been found, and a relationship between the normalized spatial Fourier Transform of the *measured* elastic scattering law and the self-distribution function has been proposed [28–30].

The presence of the above-mentioned kink in the temperature behavior of the *measured* MSD leads to two considerations. The first one (from now it represents our point a) is that the PDT [1–5] may be coincident with this type of kink, so it could be a merely instrumental effect without connections with any transition in the dynamical properties of the investigated systems. The second one (from now it represents our point b) is that, by performing different ENS measurements by varying the instrumental energy resolution, the temperature behavior of the system relaxation time can be "directly" extracted from the *measured* MSDs, i.e. $(\tau_{RES},T_{kink})$.

Finally, it should be considered that the effect of the instrumental energy resolution is not just limited on the temperature value of the PDT, but this affects the whole *measured* MDS, so it is necessary to shed a light on (from now it represents our point c) the relationship between the "*measured*" MSD and the "*real system*" MSD; this becomes very important for comparing the experimental outputs to each other and together with numerical simulations. Furthermore, this will give a new interpretation of the FSC in terms of atomic displacements and hydration-water cage-effect, and allow the calculation of the vibrational part of the MSD.

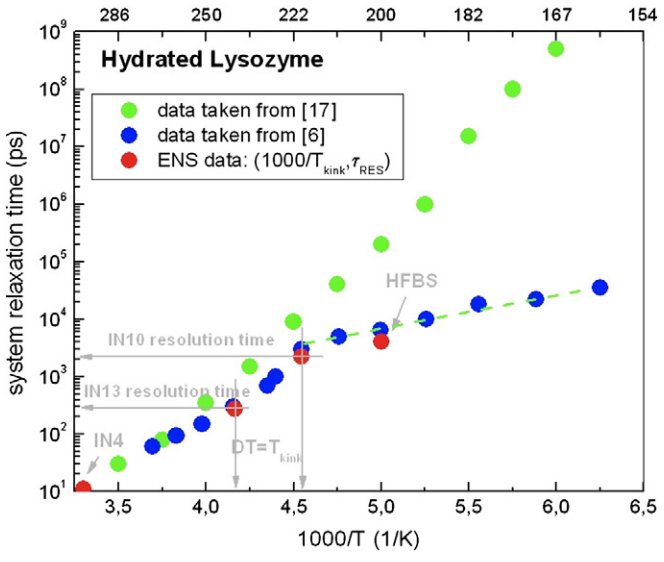


**Fig. 2.** Hydrated lysozyme (figure reproduced from ref. [40]). $D_2O$ hydrated lysozyme main relaxation times (green points) and fast relaxation times (green dashed line) have been taken from Ref. [17]; the lysozyme protein hydration water relaxation time (blue points) has been taken from Ref. [6]; finally, the red points correspond to the hydrated lysozyme relaxation time extracted from the kinks in the *measured* MSD obtained from ENS experiments performed at different instrumental energy resolution times. The horizontal arrows indicate the resolution time of IN10 and of IN13; furthermore the vertical arrows indicate the temperature values at which the system relaxation times equal the resolution times of IN10 and of IN13.

## 4. Results and discussion

In this paragraph the above-defined points, i.e. a, b, and c, are discussed and a unifying explicative hypothesis is then proposed.

### 4.1. Concerning point (a)

In Fig. 3a and b the temperature behavior of the *measured* MSD of dry and $D_2O$ hydrated lysozyme, obtained by IN10 and IN13, is respectively shown. The PDT is registered at T = 220 K in the case of IN10 and at T = 240 K in the case of IN13; moreover at T = 240 K any transition is observed in the *measured* MSD obtained by IN10, putting evidently out that the IN13 PDT temperature strictly depends on the instrumental features, i.e. instrumental energy resolution. Furthermore the PDT is registered at T = 300 K in the case of IN4, and at T = 200 K in the case of HFBS [6,16,17] — a higher energy resolution spectrometer of 0.85 μeV at the Centre for Neutron Research of the National Institute of Standards and Technology (NIST, USA). This implies that the PDT temperatures extracted in the *measured* MSDs by IN4, IN13 and IN10 are drastically influenced by the resolution effect. Proceeding in this way, a MSD *measured* at a higher resolution than the one of HFBS is needed to "check the validity" of the PDT temperature value obtained by HFBS.

Alternatively, it is possible to figure out the nature of the *measured* PDT temperatures by taking into account the system relaxation time reported in Fig. 2. In this figure, it is clearly seen that the relaxation time of the system becomes equal to the resolution time of IN4 at T = 300 K, of IN13 at T = 240 K, of IN10 at T = 220 K and of HFBS at T = 200 K (see the gray arrows). This implies that the all *measured* PDT temperatures (also the one obtained by the highest instrumental energy resolution of HFBS) occur just because the system relaxation time intersects the instrumental resolution time.

This conclusion on the nature of the PDT is also confirmed by taking into account ENS data on hydrated protein systems both (i) in the presence of bio-protectors and (ii) as a function of the hydration level *h*. These considerations are extensively reported in Ref. [40–43].

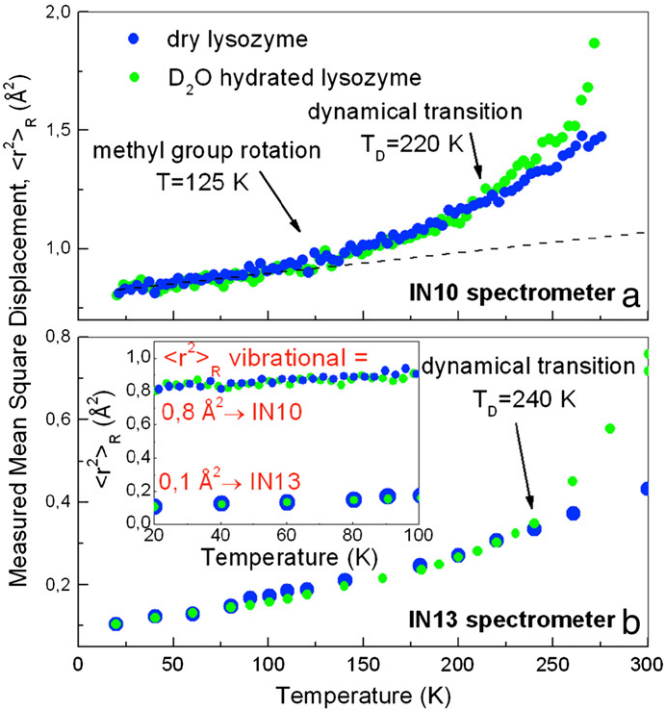


**Fig. 3.** *Measured* MSDs of lysozyme obtained using two different spectrometers with two different instrumental energy resolutions (figure reproduced from ref. [40]). Comparison between the *measured* MSD temperature behavior of dry and $D_2O$ hydrated lysozyme, obtained from data collected by both (a) IN10 and (b) IN13 spectrometers. The *dynamical transition* temperature is $T_D = 220$ K from IN10 data and $T_D = 240$ K from IN13 data. (Inset) Comparison between the dry and $D_2O$ hydrated lysozyme *measured* MSDs both from IN10 and IN13 spectrometers in the low temperature range when only vibrational motions occur. The *measured* MSD is about 0.8 Å$^2$ with IN10 and about 0.1 Å$^2$ with IN13. This discrepancy is connected to resolution effects and can be used to determine the system vibrational motion amplitudes.

Furthermore, it is important to highlight that similar results are obtained recently with other experimental techniques. More specifically, Frauenfelder et al. in Ref. [44,45] propose a new unified model of protein dynamics; more specifically, the authors show experimentally that "*the rapid increase of the MSD at ≈200 K is not a "dynamic transition"*" [44], and "*introduce a new interpretation of the Mossbauer effect in proteins and demonstrate that no dynamical transition is required […] the apparent abrupt increase in the MSD occurs when $k_\beta$ (i.e. the rate $1/\tau_\beta$ of the β fluctuations originated in the hydration shell) becomes larger than $k_{Mo}$ (i.e. the characteristic rate $1/\tau_{Mo} = 1/140$ ns of the Mossbauer effect in $^{57}Fe$)*" [45].

### 4.2. Concerning point (b)

In Fig. 2, the points ($\tau_{RES}$, $T_{kink}$) obtained by IN10 (2190 ps, 220 K), by IN13 (270 ps, 240 K), by IN4 (11 ps, 300 K), by HFBS on $D_2O$ hydrated lysozyme (2740 ps, 200 K) and by HFBS on $H_2O$ hydrated lysozyme (2740 ps, 200 K) are reported. The points ($\tau_{RES}$, $T_{kink}$), which represent the *time-scale independent* system relaxation times obtained by ENS experiments, coincide in the whole temperature range with the system relaxation time reported in Ref. [6] by Chen et al. (see Fig. 2), obtained by Quasi-Elastic Neutron Scattering. This implies that the change in the temperature behavior of the relaxation time registered at T = 220 K by Chen et al. in Ref. [6] is not a simple effect due to the employed energy resolution or to the data analysis protocol.

### 4.3. Concerning point (c)

Finally, a pertinent question arises: since the FSC represents a real transition in the dynamical properties of the studied system (as pointed out above), why is there not a signature of this transition in the *measured* MSD (which is a dynamical observable)? Answering this question can help in better understanding the nature of the FSC itself.

To go through the above-formulated problem, it is necessary to consider how the instrumental energy resolution influences the *measured* MDS, i.e. our point (c). In the self-distribution function procedure protocol [26–31,42], the relationship between the self-distribution function and the MSD is:

$$\left\langle r^2\right\rangle_R = \int_{-\infty}^{\infty} r^2 G_R^{self}(r)dr. \tag{2}$$

Starting from this relationship, it is possible to well-analyze the instrumental energy resolution effects on the *measured* MSD. In this framework, as the output of an ENS experiment is the *measured* elastic scattering law, it is:

$$\left\langle r^2\right\rangle_R = \int_{-\infty}^{\infty} FT_r\{S_R(Q,\omega=0)\}r^2\,dr \tag{3}$$

where $FT_r$ represents the spatial Fourier transform operator. By considering Eq. (1), Eq. (3) becomes:

$$\begin{aligned}\left\langle r^2\right\rangle_R &= \int_{-\infty}^{\infty} FT_r\{S_R(Q,\omega=0)\}r^2\,dr\\ &= \int_{-\infty}^{\infty} FT_r\left\{\int_{-\infty}^{\infty} I(Q,t)R(t)dt\right\}r^2dr = \int_{-\infty}^{\infty}\left\{\int_{-\infty}^{\infty} G^{self}(r,t)R(t)dt\right\}r^2\,dr\\ &= \int_{-\infty}^{\infty} R(t)\left\{\int_{-\infty}^{\infty} G^{self}(r,t)r^2\,dr\right\}dt = \int_{-\infty}^{\infty}\left\langle r^2\right\rangle(t)R(t)dt.\end{aligned} \tag{4}$$

More specifically, $\langle r^2\rangle(t)$ is the actual "system MSD", while $\langle r^2\rangle_R$ is the "*measured* MSD". The first is a function of time, whereas the second is the MSD value obtained by ENS; the latter is a pure number, not a function of time as the former, and it depends on the instrumental energy resolution of the used spectrometer. In Fig. 4a the effect on the *measured* MSD due to the instrumental energy resolution is shown: by using different energy resolutions, different *measured* MSDs correspond to the same actual system MSD. This approach is also recently adopted in Ref. [46].

It is interesting to observe what occurs in the domain of the vibrational motions, e.g. at low temperature value. When only vibrational motions occur, the actual system MSD can be considered almost constant $\langle r^2\rangle(t) \to \langle r^2\rangle^{(V)}$ (such a condition is approximately satisfied in the low temperature range up to T = 40 K); in such a case, from Eq. (4) the *measured* MSD is:

$$\left\langle r^2\right\rangle_R = \int_{-\infty}^{\infty}\left\langle r^2\right\rangle(t)R(t)dt = \int_{-\infty}^{\infty}\left\langle r^2\right\rangle^{(V)}R(t)dt = \left\langle r^2\right\rangle^{(V)}\int_{-\infty}^{\infty}R(t)dt. \tag{5}$$

Then, starting from Eq. (5), it is possible to determine the actual system MSD at the lowest temperature values. Fig. 4b shows the effect on the *measured* MSD due to the use of a different energy resolution; as can be seen, different *measured* MSDs can be associated to the same actual system MSD. A validity test of the proposed procedure is obtained by applying this procedure to the data collected on the same systems, i.e. dry and hydrated lysozyme, by the two spectrometers IN13 and IN10, working at a different energy resolution (see the inset of Fig. 3b). As a result, at the lowest temperature values we obtain the same actual system mean displacement values, i.e. 0.016 Å [42].

It is also of interest to examine the normalization procedures most frequently applied both on the *measured* elastic scattered intensity, and on the *extracted* MSD [31,42]. It is well known that the *measured* scattering law, which is a function of *Q*, represents the number of elastically diffused neutrons within a given solid angle. Therefore, normalization consisting in a multiplicative factorization, i.e. $S_R \to nS_R$, is an allowed transformation able to rescale the data since 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) to different spectra collected at different temperatures and does not produce any change in MSD evaluation. In this regard,

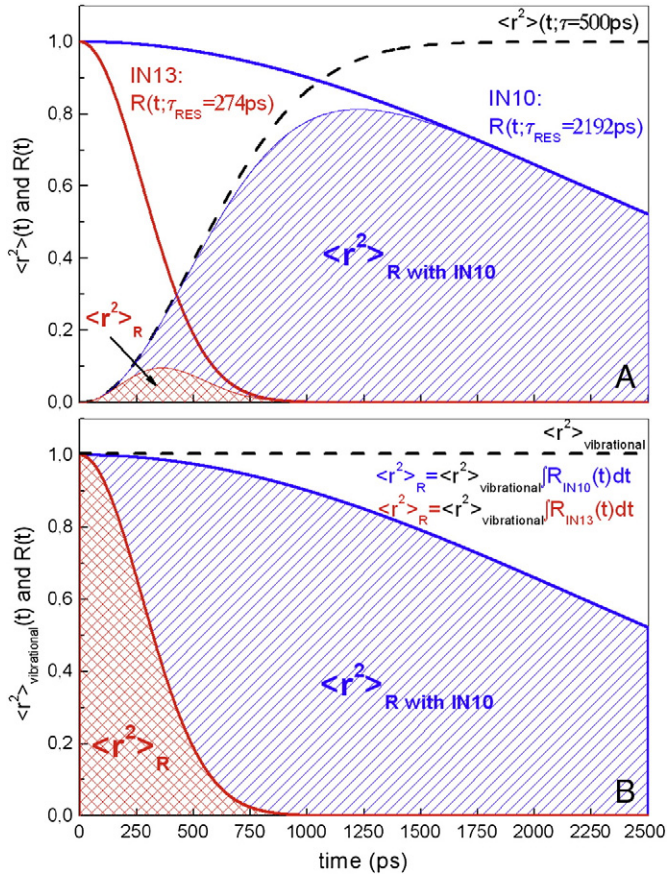


**Fig. 4.** (a) Normalized time behavior of the actual "system MSD" at a fixed $\tau$ value, $\langle r^2\rangle(t;\tau)$ (dashed curve); resolution function for different $\tau_{RES}$ values (curves), $R(t;\tau_{IN13})$ and $R(t;\tau_{IN10})$; measured MSD $\langle r^2\rangle_R$ evaluated by Eq. (4). (b) Effect on the measured MSD due to the different energy resolutions of the two spectrometers, IN13 and IN10, when only vibrational motions occur; in particular, as can be seen, different measured MSDs correspond to the same actual system MSD.

it should be taken into account that, on the contrary, the same kind of normalization applied to the logarithm of the *measured* scattering law is a misleading procedure since it leads to an 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 gives 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 a translation, this latter corresponding to a normalization of the *measured* scattering law: $Ln(S_R) + Ln(n) \rightarrow nS_R$. Finally, the normalization of the *measured* scattering law for the *measured* scattering law obtained at a given temperature (usually the lowest) is again wrong since it changes the relationship between the *measured* scattering law relative to different $Q$ values; this procedure would bring a shift of the MSD, assigning it the value of zero at the lowest temperature: $S_R(T)/S_R(T_0) \rightarrow MSD = \langle r^2\rangle_R(T) - \langle r^2\rangle_R(T_0)$. This latter is the common approach uses in the recent literature.

Having clarify the effect of instrumental energy resolution on the *measured* MSD, and having show that the "common" normalization protocol for the MSD is misleading, it is possible to come back and answer the question made at the beginning of this sub-paragraph. On that score Fig. 5 shows the *measured* MSD of both dry and $D_2O$ hydrated ($h = 0.4$) lysozyme obtained without any (low-temperature) MSD normalization. The vibrational MSD of the lysozyme in the dry state resulted to be higher than the one in the hydrated state; the latter reaches the former at a temperature value of T = 220 K that corresponds to the temperature at which the FSC is registered. These suggest that a "cage effect resulting from the hydration water on the protein

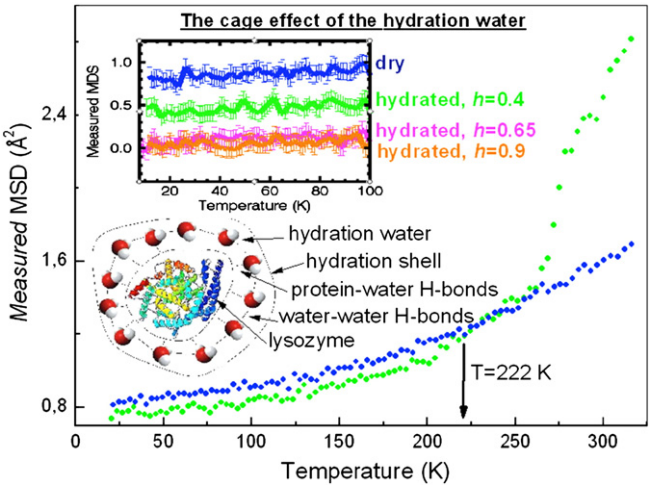


**Fig. 5.** MSDs of dry and $D_2O$ hydrated lysozyme evaluated without any low-temperature normalization. At T = 220 K, the MSD of both systems becomes equal. This could mean that a "cage effect of the water hydration shell on the protein surface" disappears at this temperature (as simply sketched in the figure). (Inset) Comparison between vibrational MSDs of dry and $D_2O$ hydrated (h = 0.4, 0.65 and 0.9) Lysozyme: moving from the dry state to the fully hydrated one, the rigidity of the protein system increases and reaches a plateau.

surface" is present at low temperatures. So, at low temperatures, the presence of hydration water has a no-intuitive effect: with this small amount of water the system is more rigid, instead to be softer. The role played by the hydration water in the increase of the rigidity of the protein system can be highlighted by studying the value of the vibrational MSD as a function of the hydration level. In the inset of Fig. 5 the vibrational MSDs of dry and hydrated lysozyme at different hydration levels are reported. As it can be seen, moving from the dry state to the fully hydrated one (i.e. h = 0.9), the rigidity of the system increases and reaches a plateau (i.e. from h = 0.65 no change are observed). It is important to highlight that, whereas at h = 0.4 the system contains only confined-like water molecules (i.e. the hydration water) at the other two higher hydration levels, i.e. h = 0.65 and h = 0.9, bulk-like water is also observed.

Finally, it is important to observe that the above-defined cage effect due to the hydration water disappears at the same temperature value of the FSC, i.e. T = 220 K. This fact suggests to link the FSC to the cage effect due to the protein hydration water: moving from low temperatures to this transition temperature, the rigidity of the hydrated system decreases and reaches the one of the dry protein, then, above this temperature, diffusion processes are occurring in the hydrated system. At T = 220 K the protein and the solvent reach a dynamical equilibrium. As a result, we may hypothesize an antithetic double role of the hydrogen-bonding network: it plays as a binding agent at low temperature, then, from T = 200 K, it allows dynamical diffusions in the system.

## 5. Conclusions

In this paper several neutron scattering experimental data obtained with different spectrometers, characterized by different accessible time-windows, on a model protein in different environments, i.e. dry and hydrated lysozyme with and without bio-protectants, are compared and analyzed by using a novel protocol in which the instrumental energy resolution effect is extrinsically took into account. This protocol together with the several accessible experimental data gives the chance to propose a complete picture of protein dynamics in which the PDT, the FSC, and the amplitude of the protein MSD are properly linked one to the others. This picture is part of my PhD Thesis, the results of which are partially reported in this paper.

More specifically, the three main points are defined and discussed. In point (a) the effect of the instrumental energy resolution on the PDT is considered. By comparing the PDTs obtained on the same system by

employing different spectrometers with different accessible time-windows, it is found that the different PDT temperatures, $T_d$, are coincident with the temperatures at which the system characteristic time intersects the instrumental resolution times. This implies that there is no connection between the above PDTs and any transition that really occur in the dynamical properties of the system.

In point (b) the correlation between the kink in the *measured* MSD and the value of the system relaxation time allows the determination of a *time-scale independent* system relaxation time by ENS. This shows at $T = 220$ K a change from Arrhenius to no-Arrhenius in the temperature behavior of the relaxation time; as a result the FSC registered by Chen et al. in Ref. [6] is a real dynamical transition, and not a simple effect due to the used energy resolution or to the employed data analysis protocol.

Finally, in point (c) a quantitative analysis of the *measured* MSD allows the determination of the vibrational part of the MSD. As a result, the vibrational MSD of the lysozyme in the dry state resulted to be higher than the one in the hydrated state; the latter reaches the former at a temperature value of $T = 220$ K that corresponds to the temperature at which the FSC occurs. These suggest that a "cage effect resulting from the hydration water on the protein surface" is present at low temperatures, then, from $T = 220$ K, the hydrogen-bonding network allows dynamical diffusion processes in the system.

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