THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

Evidence of Coexistence of Change of Caged Dynamics at T_g and the Dynamic Transition at T_d in Solvated Proteins

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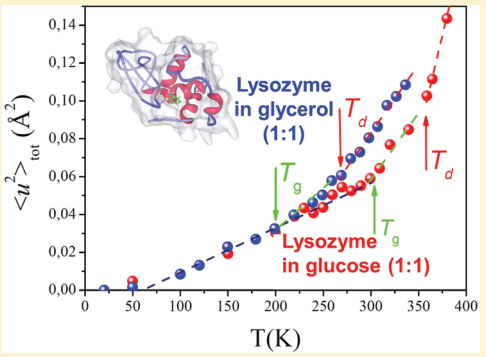
ABSTRACT: Mössbauer spectroscopy and neutron scattering measurements on proteins embedded in solvents including water and aqueous mixtures have emphasized the observation of the distinctive temperature dependence of the atomic mean square displacements, $\langle u^2 \rangle$, commonly referred to as the dynamic transition at some temperature T_d. At low temperatures, $\langle u^2 \rangle$ increases slowly, but it assumes stronger temperature dependence after crossing T_d, which depends on the time/frequency resolution of the spectrometer. Various authors have made connection of the dynamics of solvated proteins, including the dynamic transition to that of glass-forming substances. Notwithstanding, no connection is made to the similar change of temperature dependence of $\langle u^2 \rangle$ obtained by quasielastic neutron scattering when crossing the glass transition temperature T_g, generally observed in inorganic, organic, and polymeric glass-formers. Evidences are presented here to show that such a change of the temperature dependence of $\langle u^2 \rangle$ from neutron scattering at T_g is present in hydrated or solvated proteins, as well as in the solvent used, unsurprisingly since the latter is just another organic glass-former. If unaware of the existence of such a crossover of $\langle u^2 \rangle$ at T_g, and if present, it can be mistaken as the dynamic transition at T_d with the ill consequences of underestimating T_d by the lower value T_g and confusing the identification of the origin of the dynamic transition. The $\langle u^2 \rangle$ obtained by neutron scattering at not so low temperatures has contributions from the dissipation of molecules while caged by the anharmonic intermolecular potential at times before dissolution of cages by the onset of the Johari–Goldstein β-relaxation or of the merged α–β relaxation. The universal change of $\langle u^2 \rangle$ at T_g of glass-formers, independent of the spectrometer resolution, had been rationalized by sensitivity to change in volume and entropy of the dissipation of the caged molecules and its contribution to $\langle u^2 \rangle$. The same rationalization applies to hydrated and solvated proteins for the observed change of $\langle u^2 \rangle$ at T_g.



1. INTRODUCTION

Relaxation and diffusion originating from molecular motions in solvated or simply hydrated proteins naturally and ultimately are responsible for the dynamics that give rise to biological functions. In one way or the other, various approaches to understand the dynamics of proteins have been based on exploiting the similarity of the properties observed experimentally to the dynamics of glass-forming systems.[1−7] In fact, the very basic glass transition was observed in solvated proteins by calorimetry,[7−12] thermal expansion measurements,[13] and Brillouin scattering,[14] with the glass transition temperature T_g decreasing on increasing the hydration level and generally falling within the range 160−200 K. T_g can be higher if water is totally absent in the solvent such as pure glycerol or the solvent is 20 wt % of water in the disaccharide, sucrose.[12,13] Besides the relation to the glass transition, another general phenomenon exhibited by solvated proteins which has occupied much attention is the so-called dynamic transition (*i.e.*, the anharmonic onset of molecular displacements given by the mean square displacement $\langle u^2 \rangle$ at temperature T_d) observed, for instance, either by Mössbauer spectroscopy[15,16] or by neutron scattering.[7,17−34] The protein dynamic transition temperature T_d depends on the time scale or energy resolution of the spectrometer used. In the 140 ns long time limit of Mössbauer spectroscopy, the T_d measured in deoxy-myoglobin crystals is at slightly below 200 K.[15] Neutron scattering measurements with time scale ranging from about 1 ns to 15 ps show the dynamic transition occurring at higher T_d than that from Mössbauer spectroscopy. For fully hydrated myoglobin and lysozyme, T_d values from neutron scattering increase from about 210 K at a time scale of 1 ns to 250−260 at 15 ps. The explanations of the origin of the dynamic transition vary from one research group to another.[6,7,28,34−45] A recent experiment on dry and hydrated lysozyme[46] confirmed, by means of different neutron scattering experiments with different instrumental energy resolutions, that the dynamic transition observed at T_d appears when the characteristic system relaxation time intersects the resolution time and so it is

Received: June 20, 2011
Revised: December 29, 2011
Published: January 12, 2012

dx.doi.org/10.1021/jp2057892 | *J. Phys. Chem. B* 2012, 116, 1745−1757

instrument dependent. The glass transition and the dynamic transition are therefore two different processes in the same solvated or hydrated protein, and T_d is higher than T_g, in general.

In ordinary glass formers, the fast dynamics measured as a function of temperature from below to above T_g and expressed in terms of $\langle u^2(T)\rangle$ exhibit a universal behavior. As measured by the same neutron scattering technique and spectrometers including IN6, IN13, and IN16 as for the dynamic transition in solvated proteins, $\langle u^2(T)\rangle$ of ordinary glass formers has a weak and approximately linear temperature dependence for $T < T_g$, but it assumes a stronger T-dependence after crossing T_g.[47] The sensitivity of the fast process manifesting in the picosecond to nanosecond range to the glass transition is remarkable because the glass transition is affected by the structural α-relaxation when, on cooling, its relaxation time becomes too long compared with the experimental time, typically of the order of 10^3 s, and the liquid can no longer maintain equilibrium. The result is vitrification and the transformation into the glassy state. This general property found in inorganic, organic, and polymeric glass formers was touted as one of the important aspects in the dynamics of glass transition.[47−53] At temperatures below and slightly above T_g, the fast process observed at times shorter than 1 ns by neutron scattering comes from motion of molecules mutually caged by anharmonic potential. Unlike a genuine relaxation process, the loss part of the susceptibility, χ'', from the molecules while caged has no characteristic time, and hence, its dependence on frequency ω is a power law, $\chi'' \sim \omega^{-c}$, where c is a small positive number. Dielectric relaxation spectroscopy also has observed this characteristic frequency dependence of the dielectric loss $\varepsilon'' \sim \omega^{-c}$, which is generally referred to as the nearly constant loss (NCL), since $c \ll 1$.[54,55] When cages decay with the onset of the intermolecular secondary relaxation or the so-called Johari−Goldstein (JG) β-relaxation, the regime of caged dynamics and the associated NCL are terminated. It has been well established by experiments that the temperature dependence of both the dielectric strength and the relaxation time of the JG β-relaxation changes when crossing T_g[56−59] in conventional glass formers, in aqueous mixtures,[60,61] and in hydrated proteins.[41,43,60] Thus, this property of the JG β-relaxation is transferred to the NCL and is currently the only explanation that has been offered for the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g observed by neutron scattering, dynamics light scattering, and dielectric relaxation.[62,52,53]

From the similarity of solvated protein dynamics to the glass transition, it is natural to inquire whether the change in T-dependence of $\langle u^2(T)\rangle$ on crossing T_g found in ordinary glass formers has been observed or not in solvated or hydrated proteins. This question has not been addressed before, at least correctly for the experimental data of solvated and hydrated proteins, by anyone as far as we know. If indeed the change of T-dependence of $\langle u^2(T)\rangle$ at T_g has been observed in different solvated proteins, then it should be considered as another remarkable property challenging an explanation in addition to the dynamic transition. Since T_g and T_d in some cases are not far apart, if it has been observed, could it be mistaken before as the dynamic transition at T_d? We are mindful of the contribution of methyl group rotation to $\langle u^2(T)\rangle$ at temperatures higher than about 100 K in typical high-energy resolution neutron investigations.[3,34,31,63−66] The methyl group contribution makes it more difficult to identify the crossover of $\langle u^2(T)\rangle$ at T_g in cases where T_g is not much higher than 150 K. Notwithstanding, the purpose of this paper is to answer the questions posed above by re-examining published experimental data of solvated and hydrated proteins. An assist is given by the neutron scattering data of the solvents themselves by showing the presence of the change of T-dependence of $\langle u^2(T)\rangle$ at T_g and at a higher temperature T_d, in exact analogy to that observed in protein solvated by the same solvent.

2. RE-EXAMINATION OF PUBLISHED EXPERIMENTAL DATA

In the following sections, we will re-examine some already published results, coming from neutron scattering experiments done on several spectrometers and concerning proteins in different solvents or the solvent alone. We will also present a new set of data for the samples of lysozyme/glycerol and lysozyme/glucose (both at 50:50 relative weight) in an extended temperature range, which will be the starting point to propose a coherent interpretation of the experimental results. The main experimental and sample characteristics are summarized in Table 1.

Table 1. Experimental Data Sets Reviewed in This Paper, in Order of Appearance[a]

reviewed systems	instrument	ΔE (μeV)	ref
lysozyme + glycerol(D) + variable D_2O	IN13	9	19
lysozyme + glycerol(D)	HFBS	1	20
glycerol (part. deuter.)	IN13	9	67
glycerol	IN10-IN16, TOF-Stockholm	1, 200	68, 81
glycerol + H_2O (0.15 g water/g glyc)	NEAT	10−1000	44
lysozyme + D_2O 0.4h	IN16	1	24
lysozyme + D_2O (variable h)	HFBS	1	19
lysozyme + D_2O 0.4h	IN13	9	69
lysozyme + glucose (D) + variable D_2O	IN13	9	69, 70
glucose + variable D_2O	IN13	9	71, 72
disaccharides + variable D_2O	IN13	9	73−75
PM (select. deut.) + variable D_2O	IN16, IN10	1	76−78
myoglobin + D_2O 0.35h	IN6	90	28
C-PC + D_2O 0.3h	SPHERES	0.62	28

[a]The energy resolution ΔE (full width at half maximum, FWHM) is approximately linked to the longest measurable characteristic time, τ_{res}, by the relationship τ_{res}(ps) $\sim 1316/\Delta E$ (μeV).

2.1. Lysozyme Solvated in Glycerol at Different Contents of Water. These solvated lysozyme systems have been studied by two different groups.[19,20] Tsai et al. made elastic neutron scattering measurements on dry D-exchanged lysozyme/glycerol (80:20) and (50:50), as well as lysozyme/D_2O (70:30), using the neutron high flux backscattering instrument of the National Institute of Standards and Technology. The incident wavelength is at 6.271 Å with an energy resolution of 1 μeV (FWHM), picking up motions faster than about 1 ns. Paciaroni et al. performed elastic neutron scattering studies of lysozyme solvated in glycerol by using the backscattering spectrometer IN13, at the Institut Laue-Langevin, having an energy resolution 9 μeV (FWHM), which makes accessible only motions faster than about 150 ps in a spatial region smaller than ~5 Å. The samples studied

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

include the lysozyme/glycerol (50:50) as Tsai et al., and this lysozyme/glycerol mixture hydrated to the levels of 0.1*h*, 0.2*h*, 0.35*h*, 0.42*h*, and 0.83*h*, where *h* stands for grams of water/grams of lysozyme. There are neutron scattering data on the solvents alone without lysozyme. Since it has been established by experiments by several groups that the dynamics of solvated proteins are coupled to the solvent, naturally the data of neat glycerol obtained by means of IN13 by Wuttke et al.[67] and by means of IN10 and IN16 by Niss et al.,[68] and the data of glycerol/15% water by Mezei et al.[44] on the NEAT time-of-flight spectrometer, are helpful for interpretation of the data of the solvated lysozyme, as can be seen from the discussion after the data have been presented.

In Figure 1 we reproduce the data of the total $\langle u^2(T)\rangle$ of lysozyme in fully deuterated glycerol (50:50) with $0h$[20]

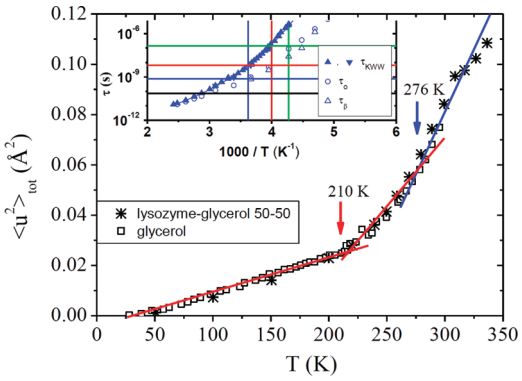


Figure 1. $\langle u^2\rangle_{tot}$ of lysozyme solvated by only glycerol taken from ref 20, with additional data at higher temperatures included to compare with the mean square displacements of pure glycerol taken from ref 67. Continuous lines are guides for eyes. In the inset are the Kohlrausch–Williams–Watts α-relaxation time, τ_{KWW}, the JG β-relaxation times obtained from a fitting procedure, τ_β, and the primitive relaxation times, τ_0, obtained from the dielectric relaxation measurements from refs 79 and 80. The green, red, blue, and black horizontal lines indicate the time scales predicted for a relaxation process originating from the dynamic transition as seen by Mössbauer spectroscopy, and IN16, IN13, and IN6 spectrometers, respectively. The green, red, and blue vertical lines indicate the reciprocal temperatures 1000/(234 K), 1000/(250 K), and (1000/276 K) where a dynamic transition has been found in pure glycerol by means of Mössbauer spectroscopy,[82] IN16,[68] and IN13,[67] respectively.

together with the mean square displacements of partially deuterated bulk glycerol $C_3H_5(OD)_3$, also measured on the spectrometer IN13 of Wuttke et al.[67] Here, $\langle u^2(T)\rangle_{tot}$ is the total mean square displacements obtained from the intensity of elastically scattered neutrons $S(Q, E \approx 0)$, as explained in ref 20. The $\langle u^2(T)\rangle$ values of lysozyme/glycerol (50:50) and pure glycerol are strikingly similar. At lower temperatures, $\langle u^2(T)\rangle$ has a nearly linear T-dependence, but on increasing temperature past $T_g \approx 190$ K of pure glycerol, it changes to a stronger T-dependence after crossing 200–210 K. As shown before in ref 47, this change of T-dependence of $\langle u^2(T)\rangle$ above T_g is a universal property of glass formers, and thus Figure 1 suggests that the same property is found in lysozyme/glycerol (50:50). The inset of Figure 1 and its relation to the main part of the figure will be discussed at the end of this subsection.

Furthermore, the similarity of $\langle u^2(T)\rangle$ shows that the protein dynamics in lysozyme/glycerol (50:50) is strongly coupled to that of the solvent glycerol, in accord with the same conclusion from investigations in other solvated proteins, such as that of the hydrated maltose binding protein by Wood et al.[25] The coupling naturally implies the possibility that the dynamic transition of lysozyme/glycerol (50:50) also can be found in bulk glycerol and at the same temperature. To look for this possibility, we need to find the common dynamic transition temperature T_d where lysozyme/glycerol (50:50) undergoes the dynamic transition and a similar "transition" of pure glycerol. Help in answering this question can be drawn by examining the data of lysozyme/glycerol (50:50) in conjunction with those of pure glycerol obtained on the IN13 spectrometer[67] reproduced separately for clarity in Figure 2,

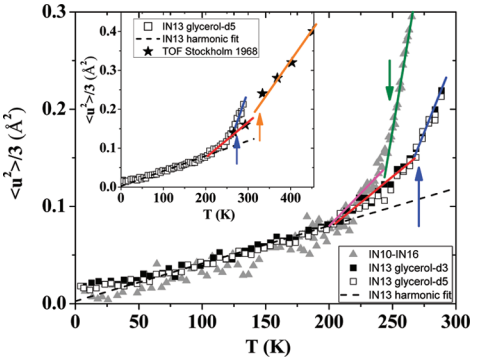


Figure 2. $\langle u^2\rangle$ versus temperature for partially deuterated glycerol (solid (d3) and open (d5) square symbols) obtained by using IN13[67] and for glycerol (gray triangles) by using IN10-IN16.[68] The dashed line is the harmonic fit for the glassy behavior of IN13 data provided in ref 67. The solid lines are guides for the eye. The green and blue arrows indicate $T_d \sim 250$ and 270 K, respectively. The inset plot compares the IN13 data for glycerol-d_5 (same symbols, lines, and arrows as in the main figure) with those (solid stars) obtained by means of the TOF Stockholm spectrometer.[81] The orange arrow is at $T_d \sim 330$ K.

where there are shown the practically identical $\langle u^2(T)\rangle$ values of the partially deuterated bulk glycerol $C_3H_5(OD)_3$ and $C_3D_5(OH)_3$. The dynamic transition of lysozyme without glycerol, hydrated at the level of 0.4 g D_2O g^{-1}, has been found by IN16 to be 220 K.[24] Since the dynamics of lysozyme solvated by glycerol is slower than that of hydrated lysozyme, and the fact that IN16 accesses motions longer than those of IN13, we can expect that T_d of lysozyme/glycerol (50:50) as estimated on the latter spectrometer will be significantly higher than 220 K. In fact, a plausible location of T_d of lysozyme/glycerol (50:50) is suggested by the arrow pointing at 276 K in Figure 1. The lines drawn do not carry any meaning other than that used to suggest a change of the T-dependence of $\langle u^2(T)\rangle$ occurring at 276 K. Moreover, the possibility that there is a change of the T-dependence of $\langle u^2(T)\rangle$ of pure glycerol occurring at 270 K is suggested by the blue arrow in Figure 2. The line in the range of higher temperatures is just a guide to indicate a stronger T-dependence, and it has no meaning because the $\langle u^2(T)\rangle$ slope is continuously increasing. Summarizing, the IN13 data of $\langle u^2(T)\rangle$ for glycerol in Figure 2 show two changes of T-dependence, one around T_g and another at higher temperature.

It is interesting to note that the $\langle u^2(T)\rangle$ values of glycerol measured with another neutron spectrometer (IN10-IN16[68]), sensitive to motions at longer time scales, show a similar scenario with two transitions: that at lowest temperature, occurring again around 200 K, and the other at highest temperature at $T_d \sim 250$ K (indicated by the green arrow). On

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745–1757

the other hand, the inset of Figure 2 shows the comparison of the IN13 data of glycerol with the data obtained on a shorter time scale (by means of the TOF Stockholm, $\Delta E \sim 200$ μeV[81]). Again a transition can be noted around 200 K, but after that, data continue with a linear behavior until $T_d \sim 330$ K, where a new regime begins.

The purported dynamic transition of lysozyme/glycerol (50:50) measured by IN13 at $T_d = 276$ K (Figure 1) has support also from the result of Tsai et al.[19] obtained in lysozyme/glycerol (50:50). Using a spectrometer with accessible time range longer than IN13, Tsai et al. found $T_d = 265$ K,[84] consistent with the slightly higher $T_d = 276$ K suggested in Figure 1, and very similar to the $T_d \sim 250$ K value found by Niss et al.[68] that used a spectrometer with a comparable resolution (shown in Figure 2). The current estimate of 276 K for T_d of lysozyme/glycerol (50:50) in Figure 1 contrasts with the much lower estimated value of $T_d = 238$ K obtained in the past[20] as the intercept between the low-T curve and the straight line approximating $\langle u^2(T)\rangle_{tot}$ at higher temperature shown again in Figure 3. However, on the basis

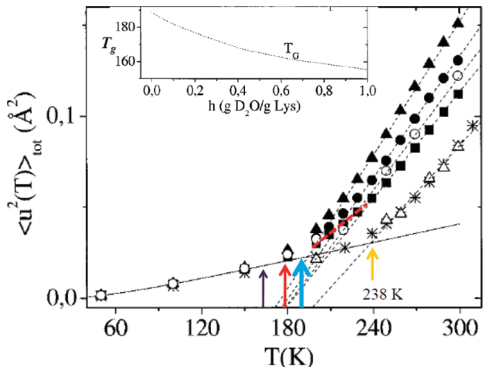


Figure 3. $\langle u^2(T)\rangle_{tot}$ versus T, for all lysozyme/glycerol (50:50) hydrated at the measured water contents of: $h = 0.0$ g D_2O/g Lys (Δ); $h = 0.1$ g D_2O/g Lys (∗); $h = 0.2$ g D_2O/g Lys (■); $h = 0.35$ g D_2O/g Lys (O); $h = 0.42$ g D_2O/g Lys (●); $h = 0.83$ g D_2O/g Lys (▲). (The solid black line represents the fit of the temperature dependence of $\langle u^2(T)\rangle_{tot}$ by a set of quantized harmonic oscillators as in an Einstein solid. The dashed black lines are linear fits to the high-temperature data. The intercept of the two lines for $h = 0.0$ g D_2O/g Lys (Δ) is at 238 K, previously considered as the dynamic transition temperature T_d in ref 20. The thick red and broken line through some data points of the sample with $h = 0.20$ is drawn to suggest T_d is about 240 K, when considered together with the black dashed straight line at higher temperatures. The inset shows the T_g of mixtures of glycerol and water as a function of h. In the main figure, the arrows from right to left indicate the T_g values of mixtures of glycerol and water are for $h = 0.0$ (light blue), 0.2 (red), and 0.42 (purple). Reprinted and readapted from ref 20 with permission from Elsevier.

of the data in an extended temperature range also from IN13 presented here in Figure 1, we have already clearly shown the presence of the transition at about 276 K. Previously, this discrepancy in the values of T_d of lysozyme/glycerol (50:50) obtained by the two groups was rationalized by the use of spectrometers with different dynamic ranges and different methods of analyses of data. This rationalization is no longer needed by rediscovering that T_d is 276 K from IN13 in Figure 1 as compared with 265 K from Tsai et al.[19]

Figure 3 shows the previous practice[20] of obtaining T_d as the intercept between the low-T solid curve and the straight line approximating $\langle u^2(T)\rangle_{tot}$ at higher temperature not only for dehydrated lysozyme/glycerol (50:50) but also hydrated to different levels of h. All the samples were prepared with fully deuterated glycerol and heavy water so that the neutron scattering comes mainly from nonexchangeable protein hydrogen atoms. The solid curve represents the fit of the temperature dependence of $\langle u^2(T)\rangle_{tot}$ by a set of quantized harmonic oscillators as in an Einstein solid.[20] The values of T_d determined in this manner are 211, 207, 202, and 160 K for $h = 0.2$, 0.35, 0.42, and 0.83, respectively. They are lower than $T_d = 220$ K for lysozyme hydrated to the level of 0.4 g D_2O g^{-1} and without glycerol found by IN16.[24] This cannot be true, because we expect that at least for hydration degrees less than or comparable to 0.4h the presence of glycerol should make the protein dynamics slower than that of lysozyme powders hydrated at 0.4h, with a consistent T_d higher than 220 K. This reasoning is also strengthened by the fact that IN16 can access longer relaxation times than IN13. On the other hand, the arrows, except the one labeled 238 K, indicate T_g of mixtures of glycerol and water for $h = 0.0$, 0.2, and 0.42 (from right to left).[20,83] Interestingly, for each hydrated lysozyme/glycerol (50:50) at level h ranging from 0.2 to 0.83, $\langle u^2(T)\rangle$ starts to rise above the solid line at temperature near T_g of the mixture of glycerol with water having exactly the same value of h. By this observation and assuming that the dynamics of hydrated lysozyme/glycerol (50:50) are strongly coupled to the solvent as in the anhydrous case shown in Figure 1 and also by other experiments,[25] we can conclude that the change of T-dependence of $\langle u^2(T)\rangle$ at T_g has also been found in each of the hydrated lysozyme/glycerol (50:50). Taking this event of $\langle u^2(T)\rangle$ at T_g into consideration, the dynamic transition occurs at a temperature T_d higher than T_g.

We have suggested that the IN13 $\langle u^2(T)\rangle$ data of pure glycerol[67] in Figure 2 show not only the change of T-dependence above T_g but also another one at a higher temperature T_c near 270 K close to the dynamic transition temperature $T_d \approx 270$ K found for the lysozyme solvated by glycerol (50:50).[20] Does this near coincidence of T_c of the solvent with T_d of the solvated lysozyme also hold in the case of hydrated lysozyme/glycerol (50:50)? The answer to this question can be given for the solvent, glycerol with 15 wt % of water, which has been studied by quasielastic neutron scattering, and $\langle u^2(T)\rangle$ determined at the 100 ps time scale comparable to that of IN13.[44] The data are reproduced in Figure 4. The blue arrow therein indicates the location of T_g determined for glycerol with 15 wt % of water (corresponding to $h = 0.18$) from the dependence of T_g on water content given by ref 83. The broken blue line connecting the few black data points suggests there is a change of T-dependence of $\langle u^2(T)\rangle$ near T_g. The black arrow indicates the higher temperature $T_c \approx$ 240 K, at which $\langle u^2(T)\rangle$ exercises another change in T-dependence. Is T_c near T_d of the $\langle u^2(T)\rangle$ of hydrated lysozyme/glycerol (50:50) at the same h? The possibility that this is indeed the case is suggested by the intercept of the two lines fitting the data of the sample with $h = 0.20$ at temperatures above and below ~240 K in Figure 3.

Let us return to the inset of Figure 1, where we extract the dielectric relaxation times, τ_α (labeled as τ_{KWW} in the figure) and τ_β of the α- and β-relaxations in the shorter time regime of pure glycerol from refs 79 and 80. As an equivalent estimate of β-relaxation times, we can also consider the primitive relaxation times τ_0 predicted by the coupling model,[53,54] that are not affected by deconvolution fitting procedures. The three vertical lines indicate locations of $1000/T$ for $T \approx 276$ K (blue), 250 K

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

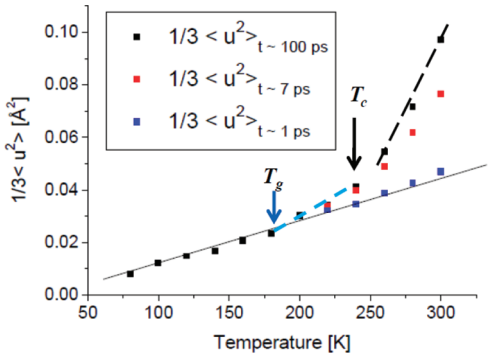


Figure 4. Temperature dependence of the mean square displacement in the mixture, glycerol/15% water, as determined for different averaging times from TOF neutron scattering data by Mezei et al.[44] The long straight line is a guide to the eye. The value of T_g is indicated by the blue arrow. The dashed blue and black lines are drawn to suggest a change of the T-dependence of $\langle u^2(T)\rangle$ at T_g and at T_c. The location of T_c indicated by the black arrow is only suggestive because the few data points available do not allow determination with accuracy. Reproduced from ref 44 by permission of IOP Publishing Ltd.

(red), and 234 K (green), corresponding respectively to T_d of lysozyme/glycerol (50:50) in Figure 1, T_c of pure glycerol from the IN10–IN16 spectrometer in Figure 2, and the value for crossover temperature obtained from Mössbauer spectroscopy,[82] which, by the way, is close to $T_d \approx 238$ K obtained by the intercept of the black dashed and continuous lines in Figure 3 for lysozyme/glycerol (50:50) in ref 20. By interpolation, we can estimate the values of τ_β or τ_0 at $T \approx 276$ K, 250 K, and 234 K to be around 800 ps, 6 ns, and 5×10^{-8} s. According to recent studies,[34] the change of the T-dependence of $\langle u^2(T)\rangle$ at T_d occurs when the time scale of a relaxation process becomes shorter than the longest time scale detectable by the neutron spectrometer (for neutron scattering τ_{res} is defined in the caption of Table 1; for Mössbauer spectroscopy, it is 140 ns). More precisely, if the relaxation process has a Lorentzian shape, the onset of the transition at T_d occurs when $\tau_{res} \sim 0.2\tau_{max}$,[34] where τ_{max} is the most probable time of the relaxation process (that could be α-, β-, or their merging). The horizontal black, blue, red, and green lines in the inset of Figure 1 indicate when the above predicted relation should occur for different experimental apparatuses, such as IN6, IN13, and IN16 spectrometers and by means of Mössbauer spectroscopy, respectively. By inspection, τ_β or τ_0 falls very close to the expected predictions. Hence, this can explain the dynamic transition at $T_d = 276$ K for solvated lysozyme and at $T_c = 270$ K for pure glycerol, both by IN13 with a time window of the order of hundreds of picoseconds and by the β-relaxation entering the window of the spectrometer on raising temperature to cross T_d and T_c. In the same way, it could explain the $T_c = 250$ K seen by Niss et al.[68] for glycerol using IN10 (Figure 2) and the very high value for transition (>330 K) seen by Larrson using a spectrometer with a very limited resolution (see the inset of Figure 2). This explanation of the dynamic transition follows the same way the phenomenon was explained in hydrated proteins, namely the water-specific or the Johari–Goldstein β-relaxation of the hydration water given before in refs 41, 42, 43, 60 and 85. There, it has been shown that, on increasing temperature, the Johari–Goldstein β-relaxation of the hydration water starts entering the time window of the spectrometer, near T_d, leading to a stronger increase of $\langle u^2(T)\rangle$ with temperature and the dynamic transition. On the other hand, τ_β at $T \approx 238$ K is $\sim 5 \times 10^{-8}$ s, which is more than a factor of 70 longer than the time window of IN13, and hence, β-relaxation of glycerol cannot cause the dynamic transition in anhydrous lysozyme/glycerol (50:50) at 238 K.[20] This is another reason for not accepting 238 K as the temperature of the occurrence of the dynamic transition.

As for the universal changes of T-dependence of the fast process at T_g measured in terms of $\langle u^2(T)\rangle$ by neutron scattering,[62] susceptibility $\chi''(\omega)$ by light scattering,[50,86] and dielectric loss $\varepsilon''(\omega)$ by dielectric spectroscopy,[55] the first question to ask is the identity of the fast process, and the second question to follow is why it is sensitive to the glass–liquid transition at T_g. Answers to these questions have been given before in refs 47, 55, and 62 and will be discussed further in section 2.5.

2.2. Hydrated Lysozyme. Sometimes the task of finding the change of T-dependence of $\langle u^2(T)\rangle$ at T_g in hydrated myoglobin and lysozyme is hampered by the contribution from the rotation of the methyl group. The relaxation time of methyl group rotation has an Arrhenius temperature dependence with an average activation energy of 10.5 kJ/mol.[34,63,66,69,87,88] Some of these neutron scattering studies have found the onset of anharmonicity in samples at all hydration levels at increasing temperature starting at $T \sim 100$–150 K and continuing to higher temperatures. Since at higher levels of hydration of the solvent, the T_g of the pure solvent as well as the corresponding solvated proteins can reach temperatures not much above 150 K;[7,89,9–14] the existence of the change of T-dependence of $\langle u^2(T)\rangle$ at T_g in these samples can no longer be ascertained. An example of this situation is presented in Figure 5 for lysozyme

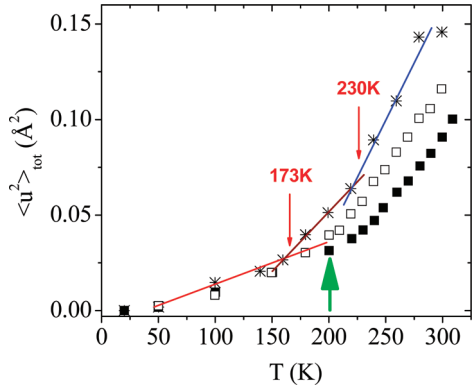


Figure 5. Total mean square displacements versus T for lysozyme at 0.4h (star), lysozyme in glycerol at 0.4h (empty square), and lysozyme in glucose 0.4h (full square) measured by IN13. Data are from ref 69. The red arrow indicates 173 K, which can be taken within uncertainty as the glass transition temperature of lysozyme at 0.4h. The lines drawn merely are used to suggest change of T-dependence of $\langle u^2(T)\rangle_{tot}$ near 173 K and near 230 K. The green arrow indicates $T_g \sim 200 \pm 5$ K of lysozyme in glucose 0.4h as well as the pure solvent glucose 0.4w itself.

hydrated at 0.4h taken from the published data of the total mean square displacement in ref 69. In the backscattering time window of IN13, the methyl group rotation contribution is significant, which makes it difficult to discriminate this process from the feature we are looking for near T_g. Certainly part of the increase in T-dependence of $\langle u^2(T)\rangle_{tot}$ above 100 K is due to methyl group rotation. By using neutron scattering with an energy resolution of 1 μeV, Roh et al. have demonstrated that the onset of anharmonicity due to methyl group rotation in dry

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745–1757

and hydrated lysozyme is at 100 K independent of hydration level.[66] Although the methyl group rotation continues to contribute to $\langle u^2(T)\rangle_{tot}$ at higher temperatures past the onset at 100 K, its contribution is expected to be smooth and cannot account for the presence of the break in the temperature dependence of $\langle u^2(T)\rangle_{tot}$ near 173 K (see Figure 5). Therefore, we can be certain that this break, which has been determined also by Brillouin scattering,[14] is due to crossing the glass transition temperature of lysozyme at 0.4*h*. Another increase in the *T*-dependence of $\langle u^2(T)\rangle_{tot}$ above ~230 K can be identified with the dynamic transition with $T_d \sim 230$ K measured by IN13. Roh et al. also found this dynamic transition in the range 200−220 K with a spectrometer that has a time resolution of about 1 ns, ten times better than that of IN13.

2.3. Lysozyme Solvated by Glucose/Water and Hydrated Glucose. The mean-square displacements of the hydrogen atoms of lysozyme solvated by deuterated glucose/water mixtures at various water contents *h* (=g D_2O/g glucose), ranging from the dry sample with 0*h* to hydrated sample with 0.15*h*, 0.40*h*, 0.60*h*, and 0.70*h*, had been measured by elastic neutron scattering using IN13 and partially reproduced here in Figure 6.[69,70] The lysozyme and deuterated glucose have equal

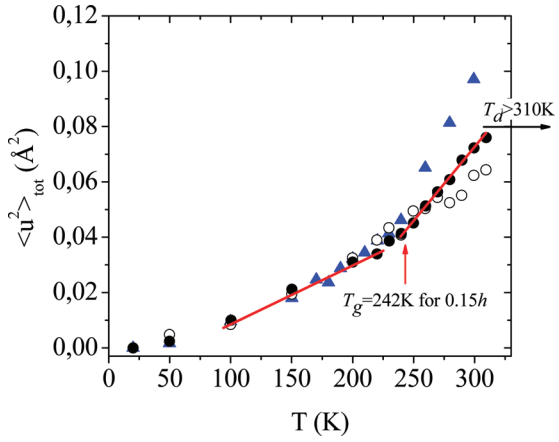


Figure 6. Total mean square displacement of lysozyme in deuterated glucose at 0*h* (○), 0.15*h* (●), 0.4*h* (▲), 0.6*h* (∗), and 0.7*h* (Δ). The arrow indicates T_g = 242 K, the glass transition temperature of the sample with 0.15*h*. The lines serve no purpose except to bring out the change of *T*-dependence of $\langle u^2(T)\rangle_{tot}$ at some temperature near T_g. Data from ref 70.

weight in the samples. The dry sample, 0*h*, as well as the hydrated samples show an onset of anharmonicity at ~100 K and continue to higher temperatures, which is attributed to the activation of methyl group reorientations intrinsic to the protein. The contributions to $\langle u^2(T)\rangle_{tot}$ from the methyl group rotation plague the task of looking for the change of *T*-dependence at T_g in all the hydrated samples except the case at 0.15*h*. This is because the T_g of the sample with 0.40*h* is ~200 K and lower for 0.6*h* and 0.7*h*, and the methyl contribution dominates $\langle u^2(T)\rangle_{tot}$ at these low temperatures. On the other hand, one clear case that is not plagued by the methyl group rotation is the sample with 0.15*h*, which has a much higher $T_g \approx 242$ K,[9,89] and the measured $\langle u^2(T)\rangle_{tot}$ rises above that of the dry sample at temperatures above ~250 K (see Figure 6), and hence the data at higher temperatures cannot come from the methyl group. Part of this rise was considered before as the signature of the dynamic transition at $T_d \approx 260$ K.[70] However, dielectric relaxation measurements of glucose with water content close to 0.15*w* have been made recently.[90] The fastest relaxation observed is from the water component in the mixture, but at 260 K its relaxation time τ_β is several orders of magnitude longer than 1 ns or 100 ps at 260 K. Thus, no relaxation process of the solvent can enter the time window of IN13 at 260 K. An estimate of the temperature at which τ_β reaches 1 ns is 310 K, and hence, T_d must be 310 K or higher, which is outside the temperature range of measurements shown in Figure 6. After eliminating both the dynamic transition and the methyl rotation as the cause of the change of *T*-dependence of $\langle u^2(T)\rangle_{tot}$ near 240−250 K, the natural choice remaining is the general property of the change of *T*-dependence of $\langle u^2(T)\rangle_{tot}$ near T_g of the sample with 0.15w.

The dynamic transition temperature T_d of the lysozyme in glucose at 0.4*h* was determined from the data of $\langle u^2(T)\rangle_{tot}$ in Figure 6 to be 220 K.[70] This value of T_d is lower than that of hydrated lysozyme without glucose by IN13 (230−240 K; see Figure 5). This is unreasonable considering that the presence of glucose in the 0.4*h* sample will slow down the dynamics, and hence, T_d of the lysozyme in glucose at 0.4*h* has to be significantly higher than 230−240 K of hydrated lysozyme with 0.4*h*. Dielectric data[90] of glucose with water content close to 0.4*h* also show τ_β is about 10^{-6} s at 220 K, and hence, the dynamic transition of the 0.4*h* sample is not at 220 K but has to be higher than 280 K, a figure obtained by extrapolating dielectric data to shorter time. By the same reasoning as given before for the 0.15*h* (0.15*w* in ref 70) sample, it seems that the change of the *T*-dependence of $\langle u^2(T)\rangle_{tot}$ near $T_g \approx 200 \pm 5$ K of the sample with 0.4*h* from dielectric relaxation[90] also exists in the data (closed triangles) of Figure 6. The reader can find in Figure 5 an unobstructed view of the same data and the change in *T*-dependence of $\langle u^2(T)\rangle_{tot}$ near $T_g \approx 200 \pm 5$ K.

Measurements of $\langle u^2(T)\rangle_{tot}$ of lysozyme in deuterated glucose at 0*h* shown in Figure 6 had been extended to higher temperatures also by IN13. These previously unpublished data are presented in Figure 7. Over the expanded temperature

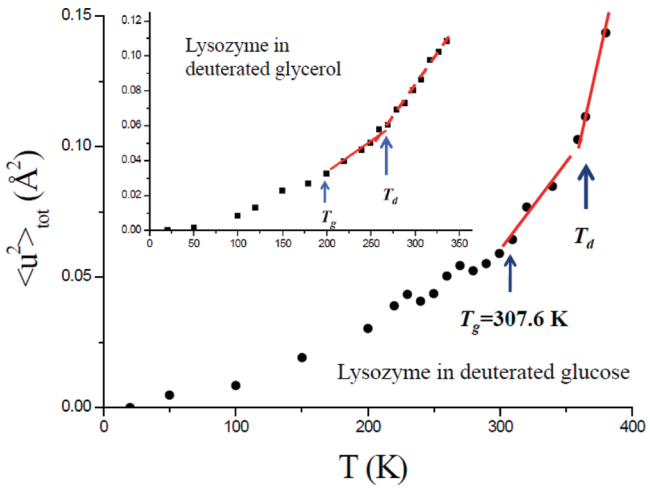


Figure 7. Total mean square displacements of lysozyme in deuterated glucose at 0*h* (●) measured up to high temperatures help to clearly identify the crossover at T_g and the dynamic transition at T_d. The inset shows the same for lysozyme solvated by deuerated glycerol.

range, $\langle u^2(T)\rangle_{tot}$ exhibits two breaks in its *T*-dependence. The one at lower temperature nearly coincides with T_g = 307.6 K of pure glucose.[91] This is where the manifestation of the change of $\langle u^2(T)\rangle_{tot}$ at T_g of lysozyme solvated by deuterated glucose can

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

be identified. Here we have assumed that the T_g of lysozyme in deuterated glucose is nearly the same as T_g of deuterated glucose, which is supported by the findings of dielectric experiments.[90] The next break in T-dependence of $\langle u^2(T)\rangle_{tot}$ can be identified with the dynamic transition which occurs at T_d near or above 370 K. The dielectric relaxation data of Kaminski et al.[91] obtained up to near 1 GHz show the secondary relaxation time of glucose is about 1 ns at 370 K. The relaxation time of this secondary relaxation is one decade longer than the IN13 time window of 100 ps. However, considering the fact that the frequency dispersion of the secondary relaxation is very broad, it is plausible that the dynamic transition is caused by the secondary relaxation entering the time window of IN13 starting from 370 K. The advantage of acquiring data of $\langle u^2(T)\rangle_{tot}$ at higher temperature for positive identification of the dynamic transition is clearly demonstrated by the example of lysozyme solvated by glucose. Had this not been done, the dynamic transition at about 370 K would be missed or mistakenly identified with the break at the lower temperature near 307 K, which turns out to be the crossover at T_g. In the inset of Figure 7, the higher temperature data of $\langle u^2(T)\rangle_{tot}$ of lysozyme solvated by deuterated glycerol help to positively identify the dynamics transition and accurately determine the value of T_d at 270 K. Although not many data points were obtained at lower temperatures, the data suggest an onset near 100 K which can be due to the methyl group rotation as found by Roh et al.

Di Bari et al.[71,72] reported IN13 elastic neutron scattering data on the monosaccharide, glucose, and its polymeric forms, amylose and amylopectin, over the hydration range h from the dry state to about 0.6 (h = g water/g dry saccharide). Their data of glucose with h = 0, 0.25, and 0.50 are reproduced in Figure 8.

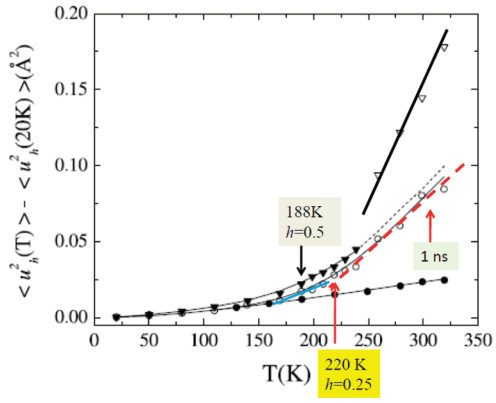


Figure 8. Total mean square fluctuation differences. Reprinted from ref 71 with permission from Elsevier. The continuous lines are fits to a harmonic Debye model for the dry sample and to the double-well potential model for the hydrated ones. The data refer to glucose samples at hydrations h = 0 (●), 0.25 (○), and 0.50 (▼). The data above 250 K for the highest hydration reported (open triangles) are obtained from the initial slope of the elastic temperature scans.

The locations of T_g for h = 0.25 and 0.50 are marked by arrows. Again, from dielectric relaxation measurements[90,92−95] of aqueous mixtures of glucose with h close to 0.25 extrapolated to higher temperatures, it is estimated that temperature higher than 300 K is needed for τ_β to reach 1 ns, which is ten times longer than the time window of IN13. Hence, the dynamic transition in the sample with h = 0.25 has not been seen in Figure 8, and the data therein actually reflect the change of the T-dependence of $\langle u^2(T)\rangle$ near T_g = 220 K. This interpretation of the data draws a distinction from the dynamic transition of Di Bari et al.[71] In their interpretation, the temperature of activation of the hydration dependent anharmonic behavior, T_a, is basically an estimate of the dynamic transition temperature T_d. The value of T_a is ≈178 K for h = 0.25, remarkably much lower than $T_d \approx$ 230−240 K by IN13 of hydrated lysozyme or myoglobin at h = 0.40[7,14] and higher at h = 0.25, without the presence of glucose. This unreasonably low value of T_a invalidates the interpretation of the dynamic transition in the sample with h = 0.25 and also in the sample with h = 0.50 having $T_a \approx$ 150 K.

At values of h comparable to those of the hydrated glucose discussed in the above, the T_d of the hydrated polymeric amylose and amylopectin should be even higher and lie outside the temperature range where $\langle u^2(T)\rangle$ data were taken, whose upper limit was nearly 340 K. Hence, the low values of T_a in the range from 210 to 150 K of amylase and amylopectin with h ranging from 0.15 to 0.60 cannot be related to the dynamic transition.

2.4. Dynamic Transition Observed at T_d and/or T_g in Hydrated Disaccharides. Elastic neutron scattering at IN13 has been used to study the dynamic properties of the aqueous mixtures of disaccharides, trehalose, maltose, and sucrose, and their mixtures in water.[73−75] The hydrated disaccharide samples have two different molar contents of water, designated by the authors as disaccharide + $6H_2O$ and disaccharide + $19H_2O$, corresponding to 0.32h and 1h, respectively. The $\langle u^2(T)\rangle$ data of all three disaccharides are similar in all respects, including temperature dependence, and the representative ones for trehalose + $6H_2O$ and trehalose + $19H_2O$ are reproduced in Figure 9.

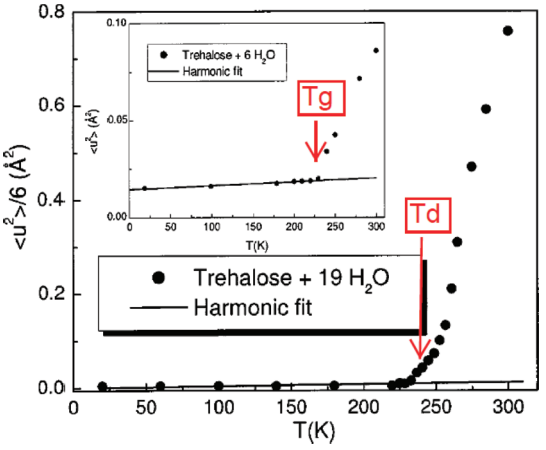


Figure 9. Figure reproduced from ref 73 showing the dependence of $\langle u^2(T)\rangle$ on temperature for trehalose + $19H_2O$ in the main figure, and trehalose + $6H_2O$ in the inset. The solid lines are the harmonic fits. For trehalose + $19H_2O$, the change of the T-dependence at $T_d \approx$ 238 K ($>T_g \approx$ 170 K) is the dynamic transition. On the other hand, for trehalose + $6H_2O$, the change of the T-dependence is at $T_g \approx$ 230 K. Reprinted and adapted with permission from ref 73. Copyright 2004 American Institute of Physics.

In refs 73 and 74 the authors gave the values 238, 235, and 233 K, for T_g of trehalose + $19H_2O$, maltose + $19H_2O$, and sucrose + $19H_2O$, respectively, and identified the onset of the rapid rise of $\langle u^2(T)\rangle$ above the harmonic response at lower temperatures to occur at T_g (see Figure 9, for example) rather than at a higher temperature T_d, which is usually the case for

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

the dynamic transition in solvated proteins or the solvents, such as in all the examples discussed before in the present paper. One of us (K.L.N.)[53] cited refs 73 and 74 for evidence of change of the T-dependence of $\langle u^2(T)\rangle$ at T_g, considering 238 K as the true T_g of trehalose + 19H_2O, and similar values for the other disaccharide + 19H_2O. Actually, after checking the values of T_g of the disaccharide + 19H_2O in the publications by Green and Angell[9] and by Bellavia et al.,[12] it becomes clear that these values quoted in refs 73−75 are far above the actual values of T_g, as obtained from calorimetric measurements. Reading off from Figure 5a−c of the paper by Bellavia et al.,[12] the correct T_g values are about 170−172 K for all three disaccharide + 19H_2O. So, how can we explain this apparent conundrum, i.e. the appearance of the onset of the rise of trehalose + 19H_2O, maltose + 19H_2O, and sucrose + 19H_2O at 238, 235, and 233 K, respectively, occurring about 65 K above T_g? One could invoke the presence of two distinctive T_g in this samples, one for the hydration water (lower) and the other for the disaccharides, but this is at odds with the accurate calorimetric measurements[12] and not common for hydrogen bonded mixtures, unless to invoke a phase separation. Actually, the simplest and more reasonable identification for these onset temperatures is that they are likely the dynamics transition temperatures of the disaccharides with 19H_2O because from the dielectric relaxation data[90] of trehalose + 6H_2O the τ_β has already reached $\sim 10^{-7}$ s at 238 K, and an increase to 19H_2O will easily take it to the time scale of 100 ps to 1 ns for detection by IN13. Recently, Gabel and Bellissent-Funel[26] have found the dynamic transition of hydrated C-phycocyanin in trehalose + 26H_2O (equivalent to 1.36h) by incoherent elastic neutron scattering using IN13 and IN16. In the temperature range from 20 to 230 K, the measured $\langle u^2(T)\rangle$ values from both spectrometers were identical and increased with temperature nearly linearly. Then, at 240−250 K the rapid increase of $\langle u^2(T)\rangle$ indicates the dynamical transition. On the other hand, the dynamical transition observed on IN16 was shifted to a slightly lower temperature than the one observed on IN13, due to the longer time window of the former.

Notwithstanding that the temperature values given in refs 73−75 for the disaccharide + 19H_2O are to be ascribed to T_d and not to T_g, the values for the disaccharide + 6H_2O (corresponding to 25 wt %) are, on the contrary, almost exactly the same as the values of T_g accurately determined from calorimetry data.[9,12] Reading off from the chart of Bellavia et al., T_g of trehalose + 6H_2O is ≈230 K, which is nearly the same temperature at which the measured $\langle u^2(T)\rangle$ values exhibit a rapid rise from the harmonic fit shown in the inset of Figure 9. Therefore, the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g is indeed observed in trehalose + 6H_2O. By the way, in mixtures of trehalose with 25 wt % of water, close to the composition of trehalose + 6H_2O, the τ_β from dielectric data[90] is about 10^{-6} s, which is more than 3 orders of magnitude longer than the time window of IN13 and, hence, cannot give rise to the dynamic transition. An extrapolation of the dielectric τ_β to higher temperature suggests that it will reach the time window of IN13 at temperatures higher than about 300 K, outside the temperature range of measurements shown in the inset of Figure 9. In this case the higher onset temperature T_d cannot be observed, as it is outside the experimentally investigated temperature range. In the case of trehalose + 19H_2O (main figure in Figure 9), the sharp rise is to be ascribed to T_d and probably overwhelms the milder change at T_g, occurring at lower temperatures.

2.5. Origin of the Change of $\langle u^2(T)\rangle$ at T_g in Neutron Scattering Experiments. The main theme of the present paper is on the presentation of evidence for the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g, preceding the dynamic transition of $\langle u^2(T)\rangle$ at a higher temperature T_d. Nevertheless, it is worthwhile to briefly mention here the origin of the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g. Despite the fact that this is a universal phenomenon found in many different classes of glass formers,[47−49,51,54] so far there is only one attempt to elucidate its origin[52,53,55,62] as far as we know. The $\langle u^2\rangle$ obtained by neutron scattering at not so low temperatures has contributions from the dissipation of excursion of molecules while caged by the anharmonic intermolecular potential at times before the cages are dissolved by the onset of β-relaxation[54] or by the merged $\alpha\beta$-relaxation. In the present case of a hydrated protein, the β-relaxation is the JG β- or water-specific relaxation of water in the hydration layer,[41−43,60] while, in solvated protein without water, the β-relaxation is the JG β-relaxation of the solvent itself, such as glycerol in Figure 1. Unlike a genuine relaxation process, the dissipation of caged molecules has no characteristic relaxation time, and hence, it appears as a "nearly constant loss (NCL)" well approximated in the susceptibility spectrum $\chi''(\omega)$ or the dielectric loss function $\varepsilon''(\omega)$ by a power law, $A\omega^{-c}$, where c is small and positive. Correspondingly, the mean square displacement, $\langle u^2(t)\rangle$, as a function of time has the power law dependence, Bt^c, with $c \ll 1$.

This feature is commonly seen in conventional glass formers, and it has been seen also in maltose binding protein (MBP) hydrated to about one hydration layer per MBP molecule by molecular dynamics simulation, as is shown in the main part of Figure 10 reproduced from Figure 2b of Wood et al.[25]

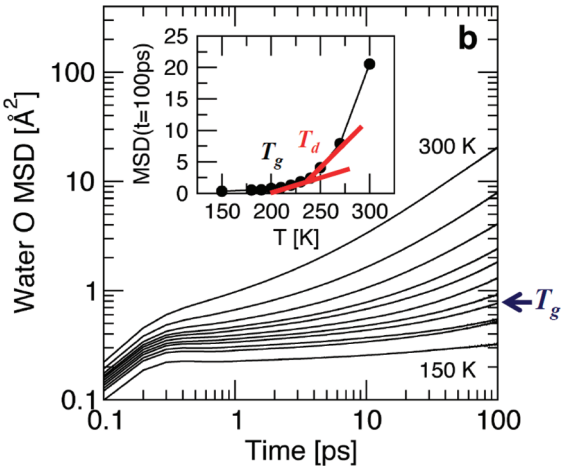


Figure 10. Time evolution of mean-square displacements of water O atoms in hydrated MBP over a range of temperature from 150 to 300 K (bottom to top: 150, 180, 190, 200, 210, 220, 230, 240, 250, 270, 300 K). (Inset) Temperature dependence of the water MSDs at 100 ps. In addition to the dynamic transition at T_d = 240 K, the MSDs show a change of T-dependence at about 200 K in the simulation, which corresponds to the 4th curve from the bottom in the main figure and is marked by the arrow . Reproduced with permission from ref 25. Copyright 2008 American Chemical Society.

Presented are the time evolutions of the mean-square displacements of water O atoms over a range of temperature from 150 to 300 K. The authors reported that 240 K is the temperature T_d of the dynamic transition in the simulations for times up to 100 ps. These times are comparable with the time

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window of the IN13 spectrometer, whose T_d = 240 K is often observed on fully hydrated proteins. The temperature dependence of the mean square displacements of the O atoms of the water molecules at 100 ps is presented in the inset of Figure 10. In addition to the dynamic transition at T_d = 240 K, the mean square displacements show a change of T-dependence at about 200 K in the simulation, which corresponds to the fourth curve from the bottom in the main figure marked by an arrow. We take this finding by simulations as another clear evidence of the presence of the change of T-dependence of $\langle u^2(T)\rangle$ at T_g.

The identification of the change of the T-dependence of $\langle u^2(T)\rangle$ of the hydration water at T_g in the purple membrane (PM) can resolve a conundrum resulting from the recent study by neutron scattering and diffraction experiments on hydrated stacks of purple membranes by Wood et al.[76,77] In this study, they examined the dynamical coupling between the PM and the hydration water by a combination of elastic incoherent neutron scattering, specific deuteration, and molecular dynamics simulations. The dynamics of hydration water were isolated by measurements on completely deuterated PM and hydrated in H_2O, while the PM dynamics were obtained from the study of natural abundance PM in D_2O. The temperature-dependence of $\langle u^2(T)\rangle$ shows changes at 120 and 260 K for the PM, and at 200 and 260 K for the hydration water. An outstanding difference in the dynamics of PM and hydration water is brought out by the presence of a change of the T-dependence of $\langle u^2(T)\rangle$ of hydration water at 200 K, in contrast to the absence of a change in the T-dependence of $\langle u^2(T)\rangle$ of PM at the same or nearly the same temperature. This observation in the membrane differs from the case of soluble proteins, where the dynamic transition can be observed by the change of the T-dependence of $\langle u^2(T)\rangle$ of either the hydration water or the protein at the same temperature. The findings in hydrated PM have cast doubt on the current view that the dynamics of the protein are coupled to the hydration water, and the two exhibit dynamical changes at the same temperature.[25,96] The words, dynamic transition a few lines above are underlined to underscore the fact that the dynamical change of soluble proteins intended by Wood et al. is apparently the change of the T-dependence of $\langle u^2(T)\rangle$ at T_d, and not at T_g. If the change of the T-dependence of $\langle u^2(T)\rangle$ of water in hydrated PM at 200 K is indeed at T_g, which we profess to be general, then there is no contradiction to the current view that those macromolecular motions respond to dynamical changes in the hydration water as far as the dynamic transition at T_d is concerned and if T_d = 260 K. This is because both water and the membrane show a change of the T-dependence of $\langle u^2(T)\rangle$ at 260 K. Corroborating evidence of this can be found from an earlier elastic incoherent neutron scattering study of dry hydrogenated PM and D_2O-hydrated hydrogenated PM using IN10 by Réat et al.[78] with energy resolution ΔE = 1 μeV and the $\langle u^2(T)\rangle$ values corresponding to motions occurring in a time shorter than 2 ns. The D_2O-hydrated hydrogenated PM shows the change in the T-dependence of $\langle u^2(T)\rangle$ or the dynamic transition at T_d near 270 K, labeled as the "solvent effect" by Réat et al. On the other hand, no such dynamic transition was found in dry hydrogenated PM. The fully deuterated PM and hydrated by D_2O studied by Réat et al. also show the dynamic transition at T_d in the range 260−270 K. All the present and previous studies of PM support that the observed change in T-dependence of $\langle u^2(T)\rangle$ near 260 K is indeed the dynamic transition of the coupled protein−hydration water, and the two exhibit dynamical changes at the same temperature T_d.

Actually there is experimental evidence that T_g of the hydrated PM studied by Wood et al. is about 200 K. Berntsen et al.[97] had studied PM hydrated to h = 0.4 and 0.2 g H_2O/g of PM by dielectric spectroscopy and differential scanning calorimetry between 120 and 300 K. They found by calorimetry a pronounced endothermic process at 190−200 K, which can be identified as T_g of the hydrated PM. By dielectric spectroscopy they found the JG β-relaxation of the hydration water with relaxation time τ_β, showing Arrhenius T-dependence at low temperatures, with activation energy of about 54 kJ/mol typical of this kind of relaxation, and changing to a stronger T-dependence at high temperatures after crossing 190−200 K. This behavior is another indication that T_g is within the range 190−200 K because the change of T-dependence of τ_β at T_g is a universal feature found in all glass formers including aqueous mixtures and hydrated proteins.[43,60,58] Since the sample studied by Wood et al. is hydrated to the level of approximately h = 0.3 g of water per g of PM, in between 0.4 and 0.2 g H_2O/g of PM in the samples of Berntsen et al., we can conclude that its T_g is within the range 190−200 K. Additional support of $T_g \approx 200$ K may be drawn from the observed onset of translational mobility in water beyond the first hydration layer indirectly observed at 200 K by monitoring the lamellar spacing of PM stacks as a function of temperature. The molecular dynamics simulation of hydration water of PM[76] has $\langle u^2(T)\rangle$ obtained at 30 ps, changing the T-dependence near 200 K (see Figure 3 in ref 76).

The dielectric relaxation data of τ_β from Berntsen et al. enable us to verify that the transition seen at 260 K in the $\langle u^2(T)\rangle$ of either the hydration water or the membrane protein is indeed the dynamic transition caused by the JG β-relaxation entering the spectral range of the IN16 with a time window shorter than about 1 ns. On extrapolating the dielectric τ_β obtained for h = 0.2 and 0.4 above 200 K by an Arrhenius law, we estimate at 260 K that τ_β is a factor of about 4 or 5 shorter than 1 ns. The actual T-dependence of τ_β is likely weaker than the Arrhenius dependence assumed because the prefactor of the latter is about 10^{-19} s and is unphysical. Therefore, a good correspondence in order of magnitude between τ_β at T = 260 K and the IN16 time resolution of about 1 ns is possible.

The fact that membrane motion does not show the change of T-dependence of $\langle u^2(T)\rangle$ at 200 K is clear. This suggests that the membrane dynamics is not as sensitive to glass transition as hydration water, probably because the presence of lipids may have a stronger impact on the membrane protein dynamics than hydration water, when the glass transition temperature is crossed. This behavior is at variance with what we have seen above (see for instance Figures 3 and 7) in some soluble proteins, where the change of the T-dependence of the protein $\langle u^2\rangle$ at T_g has been seen. However, for the dynamic transition, the dynamics of the membrane protein is still coupled to or controlled by that of hydration water and it occurs at $T_d \approx 260$ K. Thus, by identifying specifically that the 200 K transition is at T_g, the challenge of the neutron scattering data of hydrated PM on the current view of the dynamic transition is removed. Returning to the experimental fact that the power laws of $\chi''(\omega) = A\omega^{-c}$ or $\langle u^2(t)\rangle = Bt^c$, with $c \ll 1$, terminate at times of the order of τ_β, it was argued that the intensity factor A or B approximately varies with temperature like $1/\log(\tau_\beta(T))$.[62] Experimentally, it is found in conventional glass formers[98,99] as well as in aqueous mixtures[61,58,39,100] and hydrated proteins[85,14] that the relevant $\tau_\beta(T)$ changes from the Arrhenius T-dependence below T_g to a stronger T-dependence above T_g. Therefore, this change in T-dependence of $\tau_\beta(T)$ on crossing

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

T_g leads to the corresponding change in the T-dependence of $\langle u^2(T)\rangle$ at T_g. Conceptually, the change in the T-dependence of $\tau_\beta(T)$ on crossing T_g is one of the consequences of the deeper experimental fact that the α- and the JG β-relaxation are coupled or inseparable, and thus, $\tau_\beta(T)$ mimics $\tau_\alpha(T)$ in properties. One of the remarkable experimental facts showing that the two relaxations are coupled is the invariance of the ratio of their relaxation times to different combinations of pressure and temperature while keeping one of them constant.[98,99] The coupling between the two relaxations is the basic prediction of the coupling model, wherein the primitive relaxation is the analogue of the JG β-relaxation.

There are only a few papers published in the past reporting neutron scattering on D_2O hydrated proteins that have mentioned the presence of change at T_g.[24,28,101] One is the study of hydrated DNA by Sokolov et al.[101] by spectrometer with frequency resolution of ∼30 GHz corresponding to ∼5 ps in time scale. These authors integrated the quasi-elastic scattering intensities in the frequency range 60−200 GHz at different temperatures and rescaled them by the Bose factor. Although the T_g of the hydrated DNA had not been determined either by calorimetry or dielectric relaxation, it can be expected to fall within the range 180−200 K. By interpolation of the scanty results of the integrated intensity spanning across the possible T_g at 83, 138, 211, 236, 255, and 275 K, Sokolov et al. managed to show its temperature dependence changes at some temperature within 180−200 K. The data had been transformed into the frequency dependence susceptibility spectra $\chi''(\nu)$. We point out that $\chi''(\nu)$ exhibits the nearly constant loss (NCL) at all these temperatures. At 138 and 211 K, the NCL is found at frequencies lower than ∼125 GHz. At 236, 255, and 275 K, the NCL is identified with the level of the very flat minimum. When plotted against temperature, these few data points of NCL suggest that the NCL has a weak T-dependence below the purported T_g within the range 180−200 K and changes to a stronger T-dependence above it. This property, shared by NCL in many glass formers and hydrated proteins (see also Figure 10), found also in hydrated DNA reaffirms the observation that a possible break at the purported T_g is due to NCL from caged dynamics, and its sensitivity to the glass transition. Beyond addressing the possible break of integrated intensity at T_g, Sokolov et al. analyzed their data at higher temperatures of 297 and 325 K in terms of Mode Coupling Theory (MCT) and suggested there is a dynamic transition at $T \sim 230$ K, which they ascribed to the critical temperature T_c of MCT, but it cannot be the commonly known dynamic transition at T_d. The time-of-flight spectrometers they used only can sense motion shorter than ∼5 ps (like IN5) or ∼20 ps (like IN6) when extended by using 9 Å neutrons. Thus, T_d must be significantly higher than 230 K. This is because a similar system, hydrated tRNA at $h = 0.35-0.50$, has T_d near the same temperature,[102] from measurement on a spectrometer with a frequency resolution of 0.24 GHz, and time scale ∼1 ns, a hundredfold better in resolution than in ref 101. Moreover, Cornicchi et al. found T_d for DNA at $h = 0.55$ is at ∼230−240 K by IN13 with an about 10 times better resolution.[22,103]

Cursory mention of caged motion was made by Khodadadi et al.[40] in their sketch of the protein dynamics. They described the caged dynamics as fast picosecond relaxation, appearing in neutron and light scattering spectra in the frequency range ∼100 GHz to 1 THz. It is incorrect to restrict manifestation of caged dynamics to such a high frequency range. In fact, at temperatures below T_g and in some range above T_g, caged dynamics can persist down to much lower frequencies than 100 GHz and can be observed even by low frequency dielectric and mechanical relaxation as the nearly constant loss.[51,54,55] More importantly, Khodadadi et al. did not mention that caged dynamics can change temperature dependence on crossing T_g, which is the thrust of our present paper. From their view of the caged dynamics as fast picosecond relaxation is staying in the frequency range ∼100 GHz to 1 THz for all temperatures as illustrated in their Figure 11, it cannot cause any change of the T-dependence of the mean square displacement from neutron scattering at any temperature, including T_g.

Another neutron scattering study mentioning the change of the temperature dependence of the mean square displacement at T_g is on D_2O hydrated lysozyme by Zanotti et al.[24] In this study the methyl-group rotation in the lysozyme has not been taken into account, and thus, the presence of the change of the temperature dependence at $T_g \sim 150$ K is not totally certain. Most proteins fully hydrated by water alone have T_g in the range approximately from 150 to 180 K, and the complication due to methyl-group rotation contributing in the same temperature range makes it difficult to identify the presence of the break at T_g in the elastic intensity or mean square displacement. This problem is circumvented in solvated proteins using solvents with higher T_g, as shown throughout the sections above. For proteins hydrated by water alone, full or specific deuteration of the protein or choice of the spectrometer with a very short time window has to be made to eliminate or remove the methyl group rotation contribution to the observed elastic intensity in order to observe the break at T_g. This will be the subject of a paper[104] to follow this one, where the presence of a break in the T-dependence of $\langle u^2(T)\rangle$ at T_g in several proteins hydrated by water will be proven.

Besides us, Doster had shown in one recent paper the presence of two changes of the T-dependence of $\langle u^2(T)\rangle$, one at T_g and another at T_d. This he did in ref 28 from the proton mean square displacements of H_2O adsorbed to proteins obtained by SPHERE with time resolutions of 2 ns where the protein is C-PC, and by IN6 at 15 ps where the protein is myoglobin. Once more the data show that the dynamic transition temperature T_d depends on the resolution of the spectrometer, but not in the case of the changes of the T-dependence of $\langle u^2(T)\rangle$ at T_g found before in conventional glass formers.[47] The intermediate scattering function $I(Q,t)$ of adsorbed water for myoglobin at $h = 0.34$ g/g taken by IN6 shows the decay in two steps within 15 ps. The first step occurring at times less than 0.3 ps was interpreted as associated with fast hydrogen bond fluctuations dubbed the β-process with correlation time nearly independent of T and the wave vector Q, while the amplitude increases with Q and T. These fast hydrogen bond fluctuations can contribute to $\langle u^2(T)\rangle$ of caged water molecules, and its T-dependence can change at T_g if the fluctuations are sensitive to change in density on vitrification. However, this scenario applies only to hydration H_2O, and not when the solvent is glycerol, as in Figures 1−3, or glycerol/D_2O, in Figure 3. Also, this applies not to the $\langle u^2(t)\rangle$ of water O atoms over a range of temperature ranging from below T_g to some higher temperature but below T_d shown in Figure 10 for hydrated MPB. This is because the fast process seen from $\langle u^2(t)\rangle$ in Figure 10 extends to 100 ps while the hydrogen bond fluctuations according to Doster manifest at times less than about 0.3 ps. This fast process comes from caged water molecules and has an origin common to those found in

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

ordinary glass formers. It is also present in the case of the hydration water for myoglobin at h = 0.34 g/g taken by IN6, as evidenced by the plateau of $I(Q,t)$ shown for $T_g \approx 180$ and 220 K in ref 28 and certainly at temperatures below T_g, although not presented.

Mode coupling theory (MCT) also addressed caged molecular dynamics of conventional glass formers, and there is a specific prediction on the fast β-process obeying scaling relations. Doster[7,28] suggested $I(Q,t)$ of adsorbed water for myoglobin at h = 0.34 g/g taken by IN6 can be explained by the two-steps decay of MCT. Before this can be accepted, the time dependence of $I(Q,t)$ or the susceptibility minimum (obtained after Fourier transformation) and the scaling relations must be tested. Even if successful, it is not clear how MCT can address the change of T-dependence of $\langle u^2(T)\rangle$ at T_g because neither the ideal MCT nor the version that incorporates translation-rotation coupling[105,106] has predictions for the temperature dependence of the caged dynamics at or near T_g.

The crossover of the temperature dependence of $\langle u^2(T)\rangle$ of hydrated and solvated proteins at T_g has been observed before in many small molecular and polymeric glass formers by neutron scattering,[47] dynamic light scattering,[50,62] and dielectric relaxation.[51] Hence, this property of hydrated proteins could likely have the same origin as that for conventional glass formers. Proposed before as a possible molecular mechanism responsible for the crossover of the temperature dependence of $\langle u^2(T)\rangle$ at T_g of glass formers are caged molecular motions and the sensitivity of the amplitude of the motion to the change of the specific volume on crossing T_g.[62] The $\langle u^2(T)\rangle$ having this property corresponds in susceptibility to the loss of molecules moving within cages formed by the anharmonic potential at T_g. This loss has no characteristic time, and the corresponding $\langle u^2(T)\rangle$ appears as a scaleless power law or logarithmic function of time (i.e., nearly constant loss in susceptibility) and continues until the cages are dissolved by the onset of the Johari−Goldstein β-relaxation involving the motion of the entire molecule. From this relation between the caged dynamic and the β-relaxation, the change of $\langle u^2(T)\rangle$ at T_g has been rationalized[62] from the well-known and experimentally observed change of T-dependence of both the strength and the relaxation time of the β-relaxation on crossing T_g.[55,58] The abundant evidence for the change of T-dependence of the nearly constant loss when crossing T_g in common glass formers[47,50,51] could be construed to suggest the same interpretation for the solvated proteins. Notwithstanding the suggested origin of $\langle u^2(T)\rangle$ of hydrated proteins is caged dynamics, more experimental investigation is needed before a definitive conclusion can be made. In fact, another possible origin of the observed change of slope of $\langle u^2(T)\rangle$ at T_g could be vibrational contributions. Some features of molecular vibrations, including Boson peak intensity and frequency, are also sensitive to temperature change and crossing of T_g.[107] It has been demonstrated that the density of vibrational states of amorphous materials, as well as that of hydrated and solvated proteins,[14,107] undergoes a change on crossing T_g. This can affect the elastic intensity of neutron scattering, and the effect is reflected in mean square displacement. Therefore, in analyzing only data coming from elastic incoherent neutron scattering, it is difficult to single out or neglect these contributions to $\langle u^2(T)\rangle$. More experimental work with alternative techniques on hydrated and solvated proteins needs to be done before a final conclusion can be made.

3. CONCLUSION

From the evidence given in the sections above, the following conclusions can be made on the dynamics of solvated proteins probed by elastic or quasielastic neutron scattering and by molecular dynamics simulations. As a function of increasing temperature and observed from either the protein or the solvent, the mean square displacement $\langle u^2(T)\rangle$ shows a change to a stronger T-dependence after crossing temperature close to the glass transition temperature T_g, of the solvated protein. In all cases, T_g is independently determined by calorimetry or by dielectric spectroscopy. For solvated proteins, this change of $\langle u^2(T)\rangle$ at T_g coexists with the usually found dynamic transition of the solvated protein at T_d, which is higher than T_g. The change of $\langle u^2(T)\rangle$ is observed near T_g, independent of the time window of the spectrometer used for solvated proteins and conventional glass-formers. In contrast, T_d depends on the resolution of the spectrometer because the dynamic transition is caused by entrance, inside the time window of the spectrometer, of the β-relaxation of the solvent (coupled to the protein) and its most probably relaxation time is a function of temperature.

The change of $\langle u^2(T)\rangle$ at T_g was found before in many conventional glass formers of various kinds and now also in solvated proteins. This supposedly universal dynamic property of glass formers as well as in hydrated proteins poses as an outstanding problem that is challenging to explain.[62] The possible origin of this general property could be contributions to $\langle u^2(T)\rangle$ coming from vibrations including the Boson peak, as suggested by others,[107] and caged dynamics proposed by us.[47,50,51,62] Boson peak frequency and intensity[107] show changes on crossing T_g, reflected in the elastic intensity and in a possible crossover of $\langle u^2(T)\rangle$. Our explanation is the change of amplitude of caged molecular motions on crossing T_g, as has been demonstrated in conventional glass formers by different techniques (light scattering and dielectric spectroscopy), reporting a crossover in the amplitude of the nearly constant loss. More experimental work is needed to provide a conclusive explanation for the observed change of $\langle u^2(T)\rangle$ at T_g, but from the evidence presented in this paper it is clear that, regardless of its origin, a crossover does exist at T_g, it does not depend on the time scale of the spectrometer, and it occurs beside the so-called dynamic transition at T_d. Remarkably, molecular dynamic simulations of a hydrated protein have found the change of $\langle u^2(T)\rangle$ at T_g at times before the dynamic transition at T_d.

There are neutron scattering experiments of some hydrated proteins in which the presence of the change of T-dependence of $\langle u^2(T)\rangle$ at T_g is not obvious. This occurs in some hydrated proteins with methyl groups and low T_g that falls in the temperature region where rotation of the methyl group contributes strongly to the rise of $\langle u^2(T)\rangle$ with temperature. Highly hydrated proteins are another case where the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g cannot be easily observed. A plausible explanation follows from the fact found in conventional glass formers that the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g is small in strong glass formers with a low degree of cooperativity,[62] and hydration water at a high hydration level is such a case.

The dynamic transition at T_d can be mistaken as the observed change of T-dependence of $\langle u^2(T)\rangle$ at T_g if one is not aware of the presence of the latter coexisting with the former. This has happened before in the literature and has led to

dx.doi.org/10.1021/jp2057892 | J. Phys. Chem. B 2012, 116, 1745−1757

unnecessary inconsistency in the interpretation of the dynamic transition. The inconsistency is removed after taking into account of the presence of the change of the T-dependence of $\langle u^2(T)\rangle$ at T_g.

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ACKNOWLEDGMENTS

K.L.N. thanks CNR-IPCF at Pisa, Italy, and Prof. Pierangelo Rolla and Prof. Mauro Lucchesi of the Dipartimento di Fisica, Università di Pisa, Italy, for hospitality during his stay in the period from March 9th to June 1st, 2011. S.A. acknowledges the support of Galilei Ph.D. School. S.C. thanks J. Wuttke and K. Niss for making available the original published data of the mean square displacement of glycerol.

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