Protein and solvent dynamics: How strongly are they coupled?

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Analysis of Raman and neutron scattering spectra of lysozyme demonstrates that the protein dynamics follow the dynamics of the solvents glycerol and trehalose over the entire temperature range measured 100–350 K. The protein's fast conformational fluctuations and low-frequency vibrations and their temperature variations are very sensitive to behavior of the solvents. Our results give insight into previous counterintuitive observations that protein relaxation is stronger in solid trehalose than in liquid glycerol. They also provide insight into the effectiveness of glycerol as a biological cryopreservant. © *2004 American Institute of Physics.* [DOI: 10.1063/1.1764491]

I. INTRODUCTION

The influence of various solvents on biochemical activity and dynamics of proteins is a subject of active research. It is not only important for the fundamental understanding of how proteins work, but also for designing of formulations (particular solvents) for long-term preservation of proteins.[1–3] In particular, a large number of papers analyze stability and biochemical activity of proteins in various sugars and polyols.[2–6] Glycerol is generally regarded as one of the best preservants at cryogenic temperatures[7] while a disaccharide, trehalose, is widely known for its efficacy in preserving proteins at room temperature. A number of living organisms overproduce trehalose as part of a tissue preservation strategy when subjected to extremely dry conditions.[1] It has been suggested that trehalose is selected in the wild among sugars and sugar alcohols for its high glass transition temperature (T_g), and relative insensitivity of T_g to small amounts of moisture.[8]

However, a series of experimental results revealed and confirmed an unexpected behavior of proteins in trehalose as compared to proteins in glycerol. Recent light scattering data[9,10] show that at T below $\approx$270 K fast conformational fluctuations of lysozyme are stronger when protein is placed in solid trehalose than when protein is in liquid glycerol. These peculiar results are consistent with several earlier studies, including an analysis of kinetics of CO-myoglobin geminate rebinding, which showed that at $T<270$ K, local protein motions leading to escape of unbound CO are *faster* in proteins placed in glassy trehalose ($T_g \approx 400$ K) than in proteins placed in liquid glycerol ($T_g \approx 190$ K).[6] Hole burning experiments on horseradish peroxidase performed at $T \approx 4$ K demonstrate similar behavior: local protein dynamics in trehalose environment are faster than in a glycerol/water environment when both are in a solid state.[11] These unexpected observations contradict the traditional point of view that higher T_g formulations (solvents) should lead to stronger suppression of protein dynamics and activity.[8,12–15] In order to resolve this contradiction and explain the results of CO rebinding and the hole-burning experiments, the authors[6,11] assume that protein dynamics is decoupled from the trehalose-based solvent. Additionally, Sastry and Agmon[6] suggested that the slower protein dynamics in a glycerol environment were due to dehydration of the protein in the presence of glycerol.

A basic assumption underlying the traditional point of view as well as both of these explanations is that the structural (α-) relaxation of the solvent, which defines its viscosity, is the most important process for influencing the protein dynamics. In that case, trehalose is expected to impose larger friction on a protein than glycerol. However, the dynamics of glass forming systems include a variety of relaxation processes, including fast conformational fluctuations that usually occur on a picosecond time scale. They all may influence protein dynamics and activity.

The main goal of the present contribution is to explore the extent to which fast solvent dynamics influence protein motions. Our results suggest that dynamics of proteins are strongly coupled to dynamics of both trehalose and glycerol, and that the ''unexpected'' behavior discussed above appears to be a result of differences in the fast dynamics of trehalose and glycerol. Trehalose itself shows strong conformational

fluctuations even in the solid state, whereas the dynamics of glycerol on the same time scale are relatively suppressed. These results demonstrate the importance of *fast* host dynamics for protein activity and stability in highly viscous environments.

II. EXPERIMENT

Raman spectra have been measured in the backscattering geometry using a triple monochromator (Jobin Yvon T64000)[16] and an Ar laser with 514.5 nm wavelength and ≈10–20 mW power on the sample. Hen egg white lysozyme was obtained from Sigma and used without further purification. Samples were prepared with protein:trehalose mass ratios of 1:1 (L1T1) and 2:1 (L2T1), and protein:glycerol mass ratios of 1:1 (L1G1) and 1:3 (L1G3). Details of the sample preparation and Raman measurements are presented in reference.[10] Trehalose (Aldrich) was freeze dried (the same way as lysozyme-trehalose mixtures[10]) and compacted to a powder pellet. Glycerol (Fisher Scientific) was placed in an optical cell and hermetically sealed. Spectra of trehalose have a fluorescence contribution that increases strongly at low temperatures, giving rise to the rather large uncertainty in the trehalose low-temperature data. The fluorescence background correction has been described elsewhere.[10]

Neutron scattering measurements were performed on disk-chopper spectrometer at NIST, using neutrons with wavelength $\lambda=8$ Å. The spectrometer resolution was ~30 μeV (0.24 cm^{-1}). The spectra were corrected for background and summed up over all detectors in order to get better statistics. Lysozyme was washed in D_2O in order to exchange all exchangeable protons. Deuterated glycerol (Aldrich) and partially deuterated trehalose were used for lysozyme:solvent mixtures. Partially deuterated trehalose was prepared by a previously described method.[17] The level of deuteration was determined to be (63±3)% by ^1H NMR, and the sugar was recrystallized prior to use. Equal mass fractions of lysozyme and either deuterated glycerol or deuterated trehalose were dissolved in D_2O and then lyophilized. Deuterated glycerol gives negligible contribution to the neutron scattering of lysozyme:glycerol 1:1 sample. However, trehalose (because of a partial deuteration) gives ~25% of the scattering intensity of the lysozyme:trehalose 1:1 sample. Details of the neutron measurements and sample preparation will be published elsewhere.[18]

III. RESULTS

Figure 1 presents high frequency Raman spectra of the Amide I mode of lysozyme, which is very sensitive to hydrogen bonding and secondary structure of a protein.[19–21] The spectra of dry and wet lysozyme differ significantly, while the spectra of lysozyme in trehalose and in glycerol environment are similar to the spectrum of the wet protein. These results agree with earlier Raman and IR measurements of amide modes and suggest that trehalose and glycerol more-or-less replace water molecules and form hydrogen bonds with protein surface.[21] This shows that the protein in trehalose or glycerol environment differs significantly from the dry protein and it interacts strongly with the solvents.

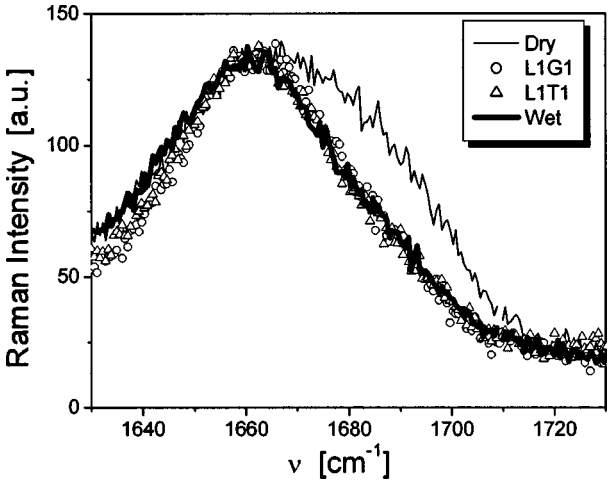

FIG. 1. Amide I mode of lysozyme at ambient temperature in dry and wet (0.4 g water/1 g of lysozyme) states compared to lysozyme in trehalose (L1T1) and in glycerol (L1G1).

Figure 2 shows low-frequency Raman spectra of lysozyme in trehalose and glycerol environments and of pure trehalose and glycerol. The data are presented as spectral density, $I_n(\upsilon)=I(\upsilon)/\{\upsilon[n(\upsilon)+1]\}$, where $n(\upsilon)=[\exp(h\upsilon/kT)-1]^{-1}$ is the Bose factor for the energy loss side. In order to estimate the solvent contribution to the spectra of protein:solvent samples we scale the spectra to high frequency modes of the solvents ($\upsilon\approx 400-550\,cm^{-1}$). The protein contribution clearly dominates the spectra in both solvents studied here (Fig. 2; see also Ref. 10). The contribution of glycerol and trehalose to the low-frequency Raman spectra of protein:solvent samples appears to be negligible.

Low-frequency spectra of all proteins and glass-forming systems have two main contributions:[22–25] (i) the quasielastic scattering (QES) that dominates the spectra at $\upsilon<15\,cm^{-1}$ and varies strongly with temperature and (ii) inelastic scattering, i.e., the so-called boson peak that appears at frequencies ≈25–50 cm^{-1}. The former is ascribed to fast conformational fluctuations while the latter is ascribed to collective vibrations[22–25] that involve the entire protein.[26]

Figure 2 demonstrates that the QES intensity is not strong in lysozyme formulated in trehalose at high T, but it decreases only slightly with decrease in temperature and remains significant even at low T [Fig. 2(a)]. The same is true for pure trehalose [Fig. 2(b)]. Dynamics of lysozyme changes drastically when it is formulated in glycerol: the QES intensity is very high at high T but decreases sharply with decrease in temperature and is strongly suppressed at low T [Fig. 2(c)]. This behavior is identical to the one observed in the spectra of pure glycerol [Fig. 2(d)]. Thus, the temperature variations of lysozyme spectra are strikingly similar to those observed in the solvents themselves.

Figure 3 shows neutron scattering spectra of lysozyme:trehalose and lysozyme:glycerol samples at two temperatures. Trivial temperature dependence [the Bose factor for the energy gain side, $n(\upsilon)$] is taken into account. The results lead to the same qualitative conclusion—Quasielastic scattering in lysozyme:glycerol is higher than in lysozyme:trehalose at high temperature, $T=320$ K, but it decreases more

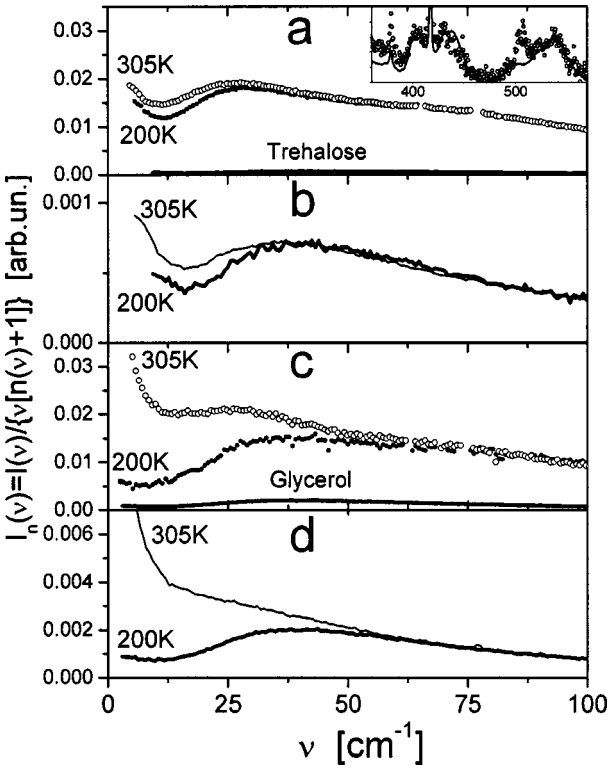

FIG. 2. Low-frequency Raman spectra of lysozyme and of pure solvents at two temperatures, 305 and 200 K. (a) Symbols—spectra of lysozyme in trehalose environment (L2T1), line—trehalose spectrum at $T=200$ K. The spectra are scaled to trehalose modes at $\upsilon\approx400-550$ cm^{-1} (see the inset). (b) Spectra of trehalose. (c) Symbols—spectra of lysozyme in glycerol environment (L1G1), line—glycerol spectrum at $T=200$ K. The spectra are scaled to glycerol modes at $\upsilon\approx400-550$ cm^{-1} (for details see Ref. 10). (d) Spectra of glycerol. All the Y axis are presented in the same units in order to emphasize that solvents give negligible contribution to the spectra of lysozyme in various environments.

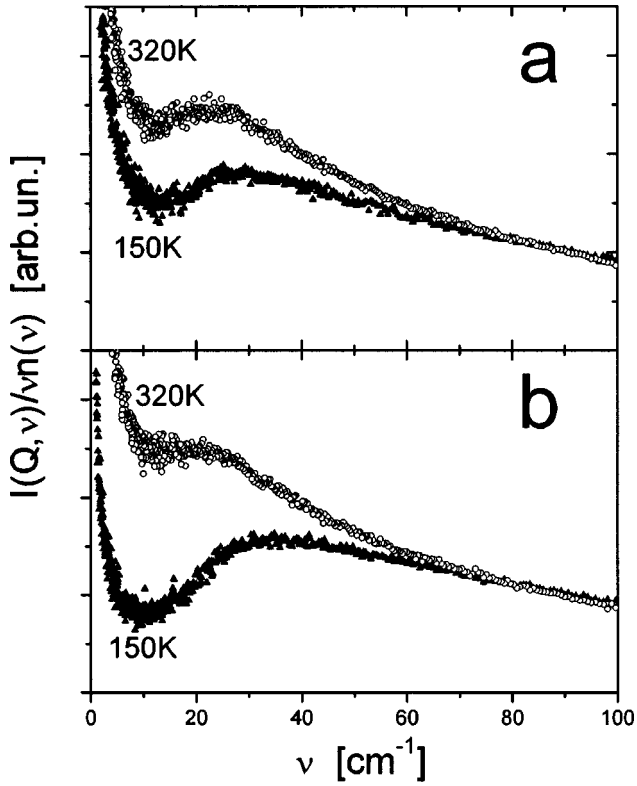

FIG. 3. Neutron scattering spectra of lysozyme:trehalose 1:1 (a) and lysozyme:glycerol 1:1 (b) samples. The spectra are summed up over all detectors and normalized by the temperature Bose factor.

strongly with decrease in temperature. As a result, the QES intensity at $T=150$ K in lysozyme:glycerol is weaker than in lysozyme:trehalose. Thus, the neutron scattering data provide clear confirmation of the light scattering results: glycerol provides superior suppression of protein dynamics than trehalose does at low temperature, while trehalose appears to be more effective at higher temperatures.

For more quantitative analysis of the light scattering data we (i) integrated the Raman intensity in the frequency range of 5–10 cm^{-1} in order to estimate temperature variations of the quasielastic scattering (QES) intensity, and (ii) fit the spectra using a simple approximation for the Raman intensity,

$$I_n(\upsilon)=\frac{A\upsilon_0}{\upsilon_0^2+\upsilon^2}+B\exp\left\{-\frac{[\ln(\upsilon/\upsilon_{\mathrm{BP}})]^2}{2[\ln(W/\upsilon_{\mathrm{BP}})]^2}\right\}, \qquad (1)$$

in order to estimate the frequency of the boson peak maximum υ_{BP}. The first term in Eq. (1) presents the QES contribution with the width υ_0 and the second term presents the boson peak approximated by a log-normal function with the width W. A log-normal distribution is the usual approximation for the asymmetric shape of the boson peak.[27–29] We should emphasize that relaxation spectra of proteins and DNA have a complex spectral shape[22,24,30] and an approximation of the QES contribution by a single Lorentzian is not appropriate for detailed analysis of relaxations. It is, however, sufficient for the purpose of our estimation of υ_{BP}.

Figure 4 shows results of the analysis. Both relaxation (the QES intensity) and vibration (the boson peak frequency) dynamics of lysozyme correlate strongly with the dynamics of the solvents. These results suggest that dynamics of proteins are strongly coupled to dynamics of both trehalose and glycerol.

IV. DISCUSSION

The influence of solvents on dynamics and activity of proteins is a subject of active investigations. Two decades ago Beece *et al.*[31] observed that solvent viscosity strongly influences most of the protein motions involved in myoglobin-CO binding reaction. Many other experimental groups have since confirmed this observation.[4,5,32] These results led many authors to speculate that higher T_g formulations should provide better biopreservation because of their higher viscosity at ambient temperature.[2,4,8,12–15] Following similar reasoning it has also been suggested that fragile glassformers should be more effective for biopreservation than strong glassformers.[12,14,15] The basis of this supposition can be explained through the Vogel-Fulcher-Tamman (VFT) equation, $\eta\propto\exp[B/(T-T_0)]$, which is used to approximate the temperature dependence of viscosity and of α relaxation in supercooled liquids. This equation predicts a divergence in relaxation times and in viscosity at some temperature T_0. T_0 is closer to T_g in fragile systems and is lower in stronger

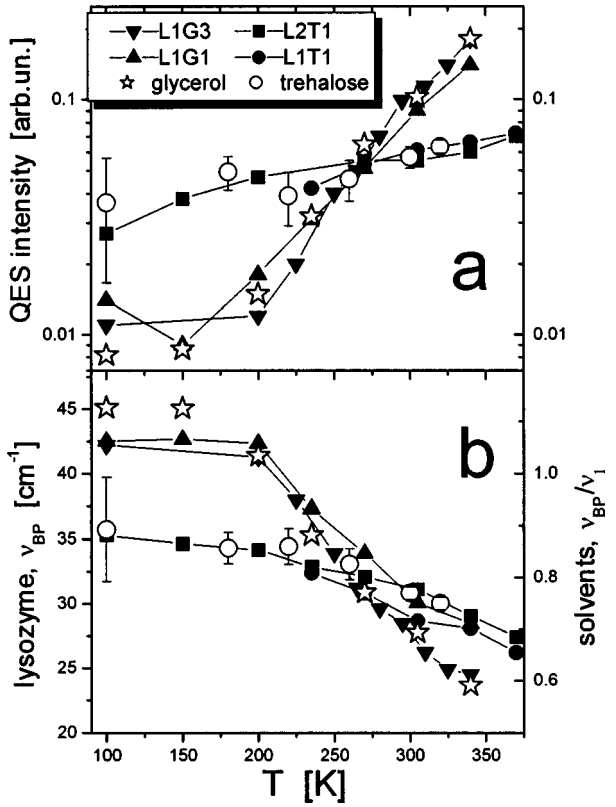

FIG. 4. (a) Temperature dependence of the normalized intensity $I_n(\upsilon)$ integrated in the frequency range (5–10) cm^{-1} for two samples of lysozyme in glycerol environments (L1G1 and L1G3), two samples of lysozyme in trehalose environments (L2T1 and L1T1), and pure glycerol and trehalose. (b) Temperature dependence of the boson peak υ_{BP} in lysozyme in different environments compared to υ_{BP}/υ_1 in pure glycerol ($\upsilon_1=40\,\mathrm{cm}^{-1}$) and in pure trehalose ($\upsilon_1=50\,\mathrm{cm}^{-1}$). Fluorescence background in trehalose spectra at low temperature is the reason for the large error bars. Error bars for all other data are much smaller. Error bars represent standard uncertainties of ± 1 standard deviation.

liquids;[33] based on this it is expected that the viscosity of the more fragile glasses will diverge, and biopreservation will be more effective, at higher temperatures.

It has been shown, however, that the solvent viscosity is not the only control parameter. Rate of biochemical reactions appears to be different in various solvents with the same viscosity.[32] These observations have been explained by a preferential hydration of a protein.[32] In that case, cosolvent is pushed out from the protein surface and viscosity on the protein surface can differ significantly from the viscosity of the bulk solvent.[34]

All these ideas are based on the assumption that the main structural relaxation (α process) that controls viscosity is the main cause for slowing down of protein's biochemical activity. However, relaxation pattern of glass forming systems includes variety of other relaxation processes and they can also affect dynamics and activity of proteins. These questions were not analyzed before. The spectra presented here (Figs. 2–4) analyze another part of protein dynamics, picosecond relaxation, and low-frequency collective vibrations. Analysis of the data (Fig. 4) shows the surprising result that dynamics of proteins follows dynamics of solvents even on the picosecond time scale. That suggests strong coupling between solvents (glycerol and trehalose) and protein, even in the glassy state.

We first discuss the behavior of the boson peak in protein and in the solvent. The nature of the boson peak vibrations in proteins remains a subject of discussion. It is observed in the light and neutron scattering spectra of all proteins and DNA.[22–25,35] Recent computer simulations[26] demonstrate that the boson peak vibrations involve the whole protein, i.e., side groups and backbone, polar and nonpolar groups. Also water of hydration is actively involved in these vibrations.[26] The nature of the boson peak vibrations in glass forming liquids is also a subject of active discussions. It is known,[36,37] however, that temperature variations of υ_{BP} in glasses follow variations of sound velocity (i.e., elastic constant). The observed correlations between variations of υ_{BP} in lysozyme and in corresponding solvents [Fig. 4(b)] suggest that the protein is coupled elastically to both trehalose and glycerol; the elastic constants of the solvent significantly affect the frequency of the boson peak in proteins.

The most interesting observation is the similarity of the temperature dependence of the QES intensity in a protein and in the solvent. The same protein shows strong or mild temperature variations of the QES intensity depending on the behavior of the solvent: It is clear from the light scattering data (Figs. 2 and 4), where solvents give negligible contributions, and also from neutron scattering spectra (Fig. 3). The observed similarity in the temperature dependence of QES is also supported by a recent neutron scattering studies of mean-squared displacement $\langle x^2\rangle$ in lysozyme placed in glycerol. It has been shown that temperature variation of $\langle x^2\rangle$ in protein placed in glycerol closely follows the temperature variations of $\langle x^2\rangle$ in bulk glycerol.[38] All these results demonstrate that protein dynamics are controlled by the solvent dynamics even on the picosecond time scale.

A remarkable result emerges from a comparison of the data at $T=200\,\mathrm{K}$ [Figs. 2 and 4(a)] at which glycerol is still in liquid state ($T\approx 1.05T_G$) and trehalose is deep in the glassy state ($T\approx T_G/2$): Glycerol and lysozyme in glycerol show low conformational activity (weak QES) while trehalose and lysozyme in trehalose demonstrate much higher activity. Thus, it appears that high conformational and biochemical activity of proteins in trehalose environment reported in Refs. 6 and 9–11 are not related to a decoupling of protein dynamics from that of the solvent, but rather to the relatively strong conformational fluctuations in trehalose even at temperatures much below its T_g. Our results also exclude a possibility of residual water being a reason for unexpectedly strong fluctuations and biochemical activity of proteins in trehalose environment. Dynamics of lysozyme in trehalose simply follows the dynamics of bulk trehalose and the latter remains active even deep in solid state.

Trehalose consists of two flexible glucose rings connected by a flexible oxygen bridge. This flexibility of the molecule means rather low energy barriers for large amplitude motions of the rings. As a result, trehalose has high conformational activity with mild temperature dependence even in the solid state when molecular diffusion is frozen on the experimental time scale [Figs. 2(b) and 4(a)]. In contrast, glycerol molecules form a strong intermolecular hydrogen-

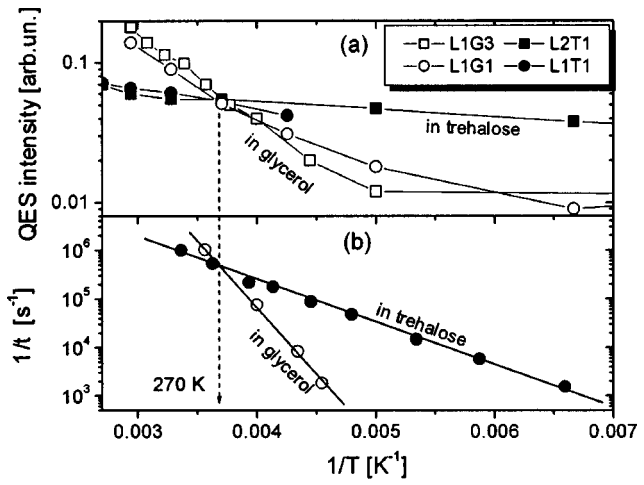

FIG. 5. (a) Integrated quasielastic intensity of lysozyme in glycerol and trehalose based formulations. (b) Inverse ligand escape time from myoglobin in glycerol and trehalose based formulations (data from Ref. 6).

bonded network leading to high energy barriers for conformational fluctuations and a strong temperature dependence of the QES intensity [Figs. 2(d) and 4(a)]. Apparently, the fast dynamics of lysozyme changes with solvent. Local conformational fluctuations of this protein in trehalose environment show mild temperature dependence [Figs. 2(a) and 4(a)] i.e., their energy barriers are low. The same protein placed in glycerol environment shows a much stronger temperature dependence of the QES intensity [Figs. 2(c) and 4(a)], indicating higher energy barriers for conformational fluctuations. This conclusion agrees with the analysis of CO-myoglobin rebinding[5,6] that the energy barriers between protein's conformational states are lower in a trehalose environment than in a glycerol/water environment. However, we ascribe this reduced energy barrier for protein motion to a relatively low energy barriers for large amplitude motions of trehalose rings. As a result, restrictions imposed by trehalose on conformational fluctuations of proteins are weaker than those imposed by interactions of a protein with a rigid glycerol structure at $T<270\,\mathrm{K}$. The latter severely limits protein motions on a short time scale and leads to an increase of effective energy barriers for conformational fluctuations of the protein in glycerol.

Figure 5 presents a comparison of the temperature variations of the QES intensity in lysozyme [Fig. 5(a)] to the rate of CO escape from myoglobin (Mb) [Fig. 5(b)] in glycerol and trehalose based formulations. We should emphasize that Fig. 5 compares two different quantities measured in different proteins: one (QES) is related to the fast picosecond relaxation and another one is related to an escape of CO group that happens on the time scale of μsec–msec.[8] Nevertheless, remarkable similarity in the temperature variation of two quantities (Fig. 5) suggests some relationship between the fast picosecond dynamics and biochemical activity of proteins. We speculate that fast relaxation of the solvent controls the fast relaxation of the protein and the latter affects its biochemical activity.

This idea gives insight into successful design of formulations based on glasses plasticized with strong glass forming solvents.[3] It has been shown that addition of a few percent of glycerol to trehalose significantly increases degradation time of proteins at room temperature. Moreover, it increases the activation energy of the protein degradation process.[3] We suspect that addition of small amount of glycerol to trehalose suppresses fast conformational fluctuations in the formulation, i.e., makes it much stronger, but keeps T_g significantly above the ambient temperature. This idea is supported by recent analysis of mean-squared displacements in trehalose/glycerol mixtures where significant depression of $\langle x^2\rangle$ has been found for mixture with ~5% glycerol content.[39] Suppression of the fast conformational fluctuations in the plasticized formulation leads to a suppression of the fluctuations and activity of the protein. As a result, degradation process is slowed down.

The idea we propose can also explain why glycerol is generally accepted as the solvent of choice for cryopreservation.[32] At low temperatures ($T<T_g\approx 190\,\mathrm{K}$), when diffusion of glycerol molecules is essentially frozen, suppression of protein dynamics and activity by glycerol is extremely effective and is far superior to trehalose. According to the classification proposed by Angell,[33] trehalose is a fragile glass-forming system while glycerol is relatively strong one. It has been shown that at $T\leqslant T_g$ fast conformational fluctuations occur with much higher probability in fragile systems than in strong systems.[25] We show here that these fast conformational fluctuations play an important role in protein dynamics, thus, the strength of the solvent (less fragile behavior) may be an important parameter for suppression of protein dynamics and biochemical activity. The stronger solvents may provide better preservation properties because of stronger suppression of the fast conformational fluctuations. These ideas contradict the proposed earlier idea[12,14,15] that more fragile systems can provide better preservation. On the other hand, this view provides one explanation for why raffinose, despite its higher T_g, provides less bioprotection than sucrose does:[40] raffinose is more fragile. Higher T_g formulations are not always better for suppression of protein dynamics and activity, and we suggest that suppression of the fast dynamics is also an important factor.

V. CONCLUSIONS

Our results suggest that proteins are strongly coupled dynamically to trehalose and to glycerol. The coupling appears in both relaxations and low-frequency vibrations of proteins. In other words, solvent dynamics control protein dynamics and activity and protein appears to be a ''slave'' of the solvents on the picosecond time scale. The presented results provide an explanation of the unusual observations reported earlier for the behavior of proteins in trehalose.[6,9–11] The reason for rather high biochemical and conformational activity of a protein in solid trehalose is not a decoupling from the environment, but rather the fragile character of trehalose. Low energy barriers for conformational fluctuations in trehalose lead to lower energy barriers for conformational fluctuations in the protein. In contrast, glycerol forms a very rigid structure that increases energy barriers for conformational fluctuations of a protein embedded in glycerol. These

results emphasize the importance of the fast host dynamics, and fast protein conformational fluctuations for biochemical activity and stability of proteins.

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