Protein dynamics in viscous solvents

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The mechanism of protein stabilization by glassy solvents is not entirely clear, and the stabilizer effective for a given protein is often discovered empirically. We use low frequency Raman spectroscopy as an effective tool to directly evaluate the ability of different solvents to suppress the conformational fluctuations that can lead to both protein activity and denaturation. We demonstrate that while trehalose provides superior suppression at high temperatures, glycerol is more effective at suppressing protein dynamics at low temperatures. These results suggest that viscosity of the solvent is not the only parameter important for biopreservation. It is also shown that glycerol and water enhance the high temperature conformational fluctuations relative to dry lysozyme, which explains the lower melting temperatures T_m in the hydrated protein and protein formulated in glycerol. © *2003 American Institute of Physics.* [DOI: 10.1063/1.1541614]

INTRODUCTION

Extreme temperatures and/or dehydration irreversibly damage most biologically active organisms. However, a limited number of plants and organisms can withstand long periods of dehydration while others retain biological activity after experiencing temperatures well below the freezing point of water. A crucial biochemical commonality among these life forms is the accumulation of viscous sugars or polyalcohols in the intra- and intercellular fluids.[1] Upon dehydration or cooling, these viscous liquids are believed to vitrify and kinetically arrest diffusion and restrict molecular motion. This prevents the osmotic stresses from destroying cell membranes, inhibits denaturation of both periplasmic and cytoplasmic proteins, and even frustrates ice formation. The kinetic hindering of these processes allows the life form to remain dormant until the temperature and/or moisture conditions are once again favorable for biological activity.

Likewise, protein-based pharmaceuticals are fragile in that they require stabilization against dehydration and thermal extremes to preserve biological function. However, the molecular mechanisms by which a viscous sugar or polyalchohol cosolvent confers stability are poorly understood. Stabilization is realized through a balance of solvent viscosity, protein conformation, dynamics, and specific interactions between the protein, the solvent, and water. There is a general understanding that reduced molecular mobility and glass formation is important for preservation. However, glass formation alone does not ensure preservation. Even in cases where the capacity for hydrogen bonding exists, certain glasses provide more effective protection than others upon freeze-drying.[2–4] Thus, a deeper understanding of how proteins respond to different glassy environments is required.

It is known that low-frequency Raman spectra of proteins[5,6] and DNA (Ref. 7) are very sensitive to variations of temperature and environment of biopolymers. In this study we use low-frequency Raman spectroscopy to directly probe the dynamical differences of the protein lysozyme preserved in both glycerol and trehalose. These are two well-known and widely used cryoprotectants for lyophilizing proteins. It was recently shown that glycerol and trehalose have very different effects on a protein function both deep in the glassy state and above the glass transition temperature T_g,[8] as well as in an aqueous environment.[9] We identify strong differences in the manner by which glycerol and trehalose affect the picosecond dynamics of lysozyme over a wide range of temperatures. These differences correlate strongly with established trends in the biochemical activity and denaturation temperatures.

MATERIALS AND METHODS

Dry chicken egg white lysozyme powder was obtained from Sigma (Certain commercial equipment and materials are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology nor does it imply the material or equipment identified is necessarily the best available for this purpose) and used without further purification. Thermogravimetric analysis (TGA) shows that content of water is below 4% by weight in the dry sample. At this level of hydration, the water is bound principally to the charged groups.[10] Wet lysozyme was produced by exposing the lysozyme powder for 3 weeks to 98% relative humidity at room temperature. Protein took up additionally ~0.35 g water per 1 g of lysozyme. That corresponds to ~1:280 protein/water mole ratio and is considered as protein having a complete water monolayer.[10] Stabilized aqueous solutions were created by dissolving equal mass fractions (~1:155 mole ratio) of dry lysozyme powder

and glycerol (L1G1) as well as lysozyme:trehalose mixtures at mass ratios of 1:1 (~1:38 mole ratio) (L1T1) and 2:1 (~1:19 mole ratio) (L2T1). These aqueous solutions were freeze-dried into powders and maintained at subambient temperatures. A sample of lysozyme:glycerol with 1:3 mass ratio (~1:465 mole ratio) (L1G3) was prepared by directly mixing dry lysozyme with glycerol. Crystallographic structure of wet lysozyme is known, however, no comparable structural information on lysozyme in glycerol or trehalose is available. It is known that both glycerol and trehalose replace the surface water and forms hydrogen bonds with protein surface, and protein (lysozyme) remains intact (properly folded).[11,12] In particular, Infrared measurements of Amide modes[11] demonstrate that trehalose forms hydrogen bonds with lysozyme similar to water hydrogen bonds. Our Raman measurements on lysozyme in trehalose and in glycerol (samples L1T1, L2T1, L1G1, and L1G3) also show no significant spectral differences compared to the wet lysozyme at the Amide I region (data not shown).

Low frequency Raman measurements were performed on samples (0.3–0.5) mm thick sealed between sapphire windows. Sapphire does not have a significant contribution to the Raman signal in the low-frequency region ($\nu < 100\ \mathrm{cm}^{-1}$) of interest. An optical cryofurnace (Janis ST100) was used for the temperature variations. At least two separate samples were prepared for each case, and multiple measurements were performed on each sample to ensure reproducibility. Significant differences were not observed.

The Raman scattering spectra were measured in the backscattering geometry using a triple-monochromator Jobin Yvon T64000 spectrometer with a 514.5 nm Ar^{++} laser and (10–25) mW of power incident on the sample. Special care was taken to avoid a contribution of the tail of the elastic line in low frequency ($\nu < 10\ \mathrm{cm}^{-1}$) region. The measured signal has two contributions: Raman scattering and fluorescence. The latter has featureless spectrum (approximated by a second order polynomial) that was subtracted from the raw spectra. All the data presented here have been corrected for the fluorescence. The ratio of the Raman signal to the fluorescence background depends on sample and varies strongly with temperature. The fluorescence background was considerably weaker than the Raman signal at temperatures above 200–250 K, and the fluorescence correction does not introduce significant uncertainty into the data. However, the fluorescence contribution increases strongly below 200 K. Our estimates show that the maximum uncertainty in the low-frequency Raman intensity (due to the fluorescence correction) is ±20% at $T = 100$ K, but this decreases sharply with increasing temperature. Reliable low-frequency Raman measurements were not possible below 100 K due to the strong fluorescent background. With L1T1 samples the fluorescence background (mostly due to trehalose) was already significant at 250 K, and data for these samples are not presented below 225 K.

RESULTS

To isolate and study the dynamics of the lysozyme itself, one must first determine the contributions of the pure solvent to the total Raman spectrum. Figure 1 compares the Raman spectra of L1G1 and pure glycerol. These spectra are scaled at the high-frequency modes of glycerol (~400 cm^{-1} to 550 cm^{-1}) to estimate the contribution of the glycerol to the total spectrum of the L1G1 sample. In the low frequency range of interest, $3\ \mathrm{cm}^{-1} < \nu < 100\ \mathrm{cm}^{-1}$, the scattering from pure glycerol is negligible and the Raman spectrum is dominated by the lysozyme. A similar analysis reveals that contribution of glycerol to the low-frequency Raman spectra of L1G3 is also weak. Figure 2 shows the Raman spectra of L1T1 and pure trehalose scaled at the high-frequency modes of trehalose (380 to 550 cm^{-1}). The contribution of trehalose in the low frequency spectrum is also negligible. Estimating water's contribution to the Raman spectra of the wet

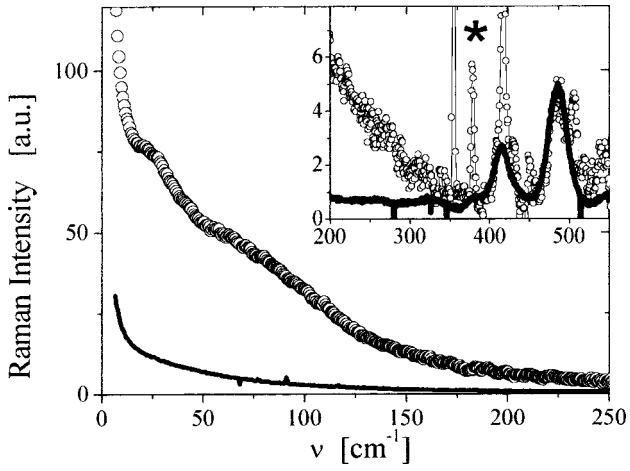

FIG. 1. Low-frequency Raman spectra of lysozyme:glycerol sample (L1G1) (symbols) and glycerol (lines) at 340 K. The inset shows high-frequency part of the spectra. The intensities of the spectra are scaled to glycerol modes at $\nu \approx 400$ to 550 cm^{-1}. The asterisk marks Raman modes of sapphire.

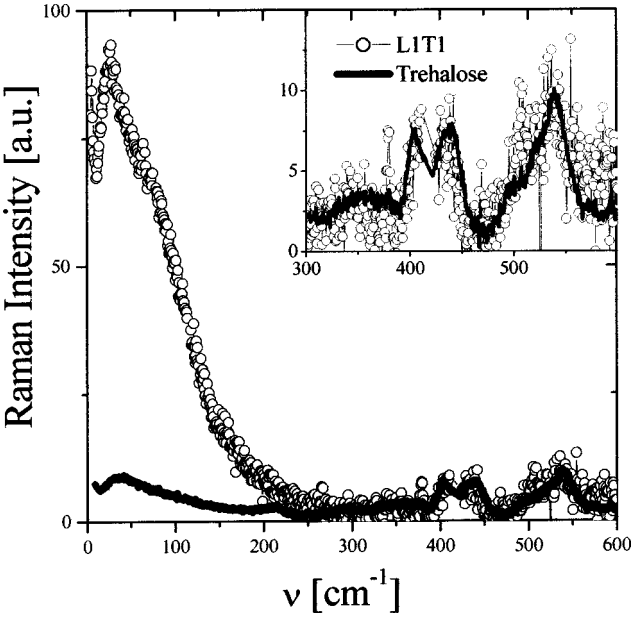

FIG. 2. Low-frequency Raman spectra of lysozyme:trehalose sample (L1T1) (symbols) and trehalose (lines) at room temperature. The inset shows high-frequency part of the spectra. The intensities are scaled to trehalose modes at $\nu \approx 380$ to 550 cm^{-1}.

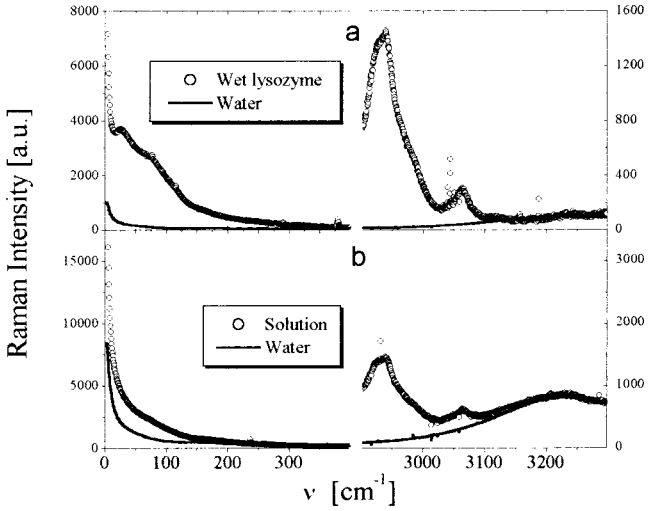

FIG. 3. Comparison of Raman spectra of wet lysozyme and water (a) and of solution (1 g of protein/3 g of water) and water (b) at room temperature. The intensities of the spectra are scaled to high frequency mode at $\nu \approx 3100$ to 3400 cm^{-1}.

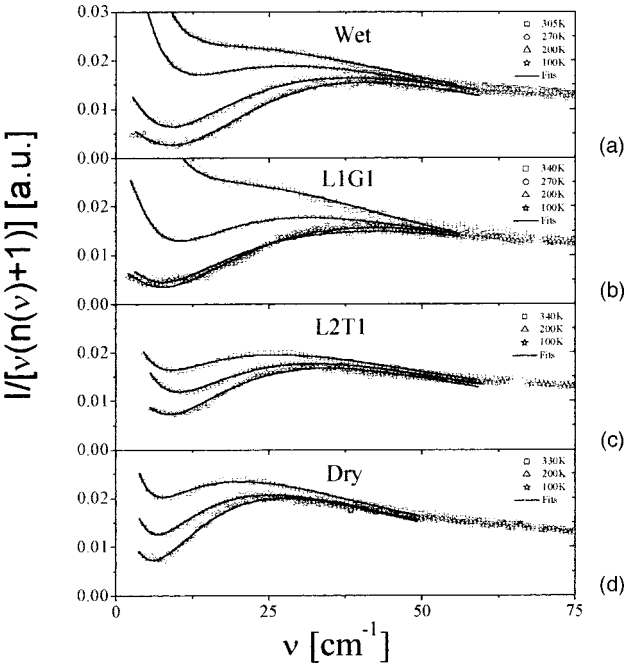

FIG. 4. The low-frequency Raman spectra of wet lysozyme (a), lysozyme/glycerol sample (L1G1) (b), lysozyme/trehalose sample (L2T1) (c), and dry lysozyme (d) at different temperatures.

lysozyme is more complicated due to absence of sharp water modes in the Raman spectra. Figure 3(a) shows a comparison of the Raman spectra of wet lysozyme and bulk water. The spectra are scaled at the high frequency O–H mode (3100–3400 cm^{-1}). The contribution of water at low frequencies is below 10% of the total signal. In addition, Fig. 3(b) shows that contribution of water to the low-frequency Raman spectra is below 50% even in concentrated lysozyme solution (1 g of protein/3 g of water) where there is nearly 10 times more water than in our "wet" sample. This confirms our conclusion that contribution of water to the Raman signal of wet lysozyme is below 10%. Furthermore, it is worth emphasizing that 20 of the 129 amino acid residues in lysozyme contain hydroxyls that will contribute to the Raman spectra in the 3100–3400 cm^{-1} region. Thus, comparisons based on scaling the –OH band overestimate the contributions from the pure water. Recently Urabe *et al.*[6] interpreted the quasielastic scattering (spectra below ~15 cm^{-1}) in wet lysozyme as direct scattering of light on water of hydration. Our results (Fig. 3) show that this interpretation is not correct and contribution of water molecules to the low-frequency Raman spectra of wet lysozyme is negligible. The present analysis (Figs. 1–3) suggests that the protein dominates the low-frequency Raman spectra in all of our samples, and analysis of the spectra provides information on protein dynamics.

Figures 4 and 5 present the low-frequency Raman spectra as a function of temperature for the lysozyme in the different solvents and the dry state. The spectra are presented in terms of spectral density, $I_n = I/\{\nu[n(\nu)+1]\}$, and normalized over the frequency range of 75–150 cm^{-1}, where harmonic vibrations dominate. In such a spectral density plot, the scattering intensity is divided by the frequency ν and scaled by the Bose population factor $n(\nu)+1=[1-\exp(-h\nu/k_BT)]^{-1}$ to account for trivial temperature differences (in the classical high temperature limits where $h\nu \gg k_BT$, $I_n \approx I/k_BT$). In a purely harmonic system, Bose scaling results in spectral densities that are independent of temperature. This temperature independence is not observed at the lower frequencies in Figs. 4 and 5, emphasizing that the motions are more complicated than simple harmonic vibrations.

The low-frequency Raman and neutron scattering spectra of proteins contain two primary components:[13–15] (i) an

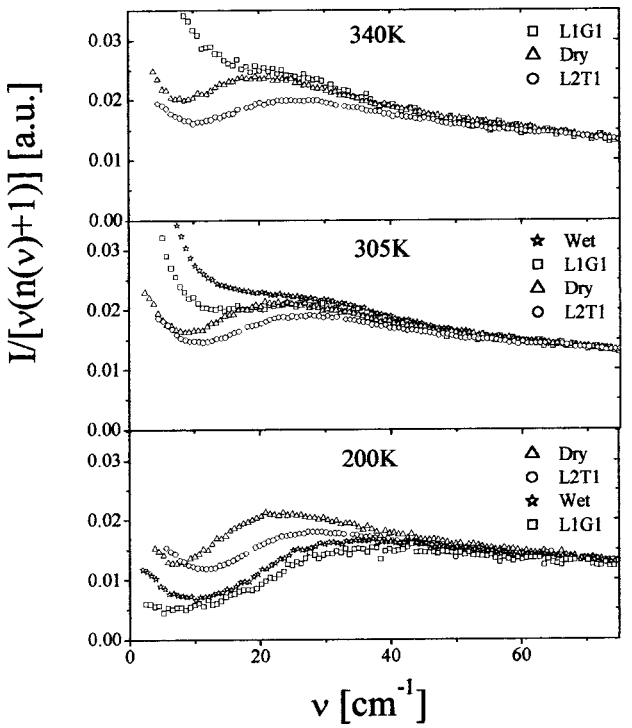

FIG. 5. The low-frequency Raman spectra of lysozyme in different solvents at three selected temperatures. The intensities are normalized at frequencies $\nu \approx 75$ to 150 cm^{-1}.

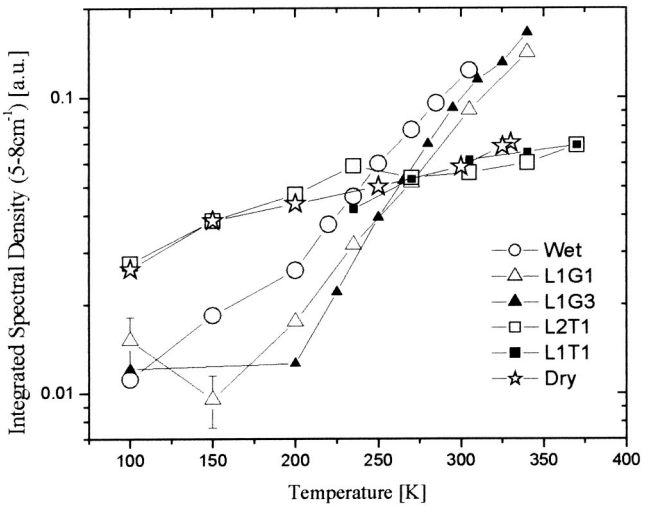

FIG. 6. Normalized Raman intensity $I_n(\nu)$ integrated over the frequency range 5 to 8 cm^{-1} for different lysozyme samples.

inelastic peak at $\nu \sim 10-50$ cm^{-1}, commonly referred to as the boson peak, and (ii) a quasielastic scattering (QES) or broadening around elastic line, prominent for $\nu < 15$ cm^{-1}. The former corresponds to low-frequency collective amino-acid residues vibrations related to the elasticity of the protein while the latter reflects local conformational jumps or fast conformational fluctuations. A higher QES intensity corresponds to greater protein flexibility and faster local rearrangements of the amino-acid residues.

Both the boson peak and QES are clearly visible in the spectra of Fig. 4. The main variations are (i) an increase in the QES intensity and (ii) softening of the boson peak with increasing temperature. Both variations are strong in wet lysozyme and lysozyme/glycerol samples but mild in the dry and lysozyme/trehalose samples. In particular, the quasielastic intensity increases more than ten times for the proteins dissolved in water and glycerol, while only a factor of 2 is observed in the dry and trehalose preserved proteins.

Comparison of the spectra of different samples (Fig. 5) demonstrates the peculiar influence of water and glycerol on dynamics of lysozyme. The quasielastic scattering in the wet and in the glycerol preserved samples is much weaker than in the dry sample at low T (<250 K), while it is much stronger at higher T. In contrast, the spectra of lysozyme/trehalose samples are similar to the dry lysozyme with slightly higher frequency of the boson peak maximum, $\nu_{\max}$. The quasielastic intensity, however, increases more slowly with temperature, especially at higher T.

ANALYSIS OF THE SPECTRA

As a first approach, we simply integrate the quasielastic intensity as a model independent measure of conformational activities of the protein. Figure 6 summarizes the I_n integrated over $\nu = (5-8)$ cm^{-1} as a function of temperature. This part of the spectra is dominated by the QES contribution. At 100 K, both water and glycerol substantially suppress the QES intensity in lysozyme, more so than either the trehalose embedded or dry conditions. A rapid increase in the QES intensity occurs near 150 to 200 K for the wet and glycerol based samples. There is a crossover and the QES intensities exceed that of the dry lysozyme near 250 K and 280 K for the wet and glycerol environments, respectively. Interestingly, the QES intensity in the lysozyme:trehalose samples is similar to the dry lysozyme over the entire temperature range. In both, the QES increase with temperature is subtler than in either the wet or lysozyme:glycerol samples.

For a more quantitative analysis, these spectra are typically fit by a sum of the vibrational (the boson peak) and relaxation (QES) contributions. The latter, in most cases, is approximated by a multiple Lorentzian function.[6,16] For example, the Raman spectra of lysozyme were analyzed[6] assuming five vibrational modes and two relaxation processes described by Lorentzians (a total of 19 free fitting parameters). It was found[12,14–16] however, that the relaxation spectra of biopolymers are rather complex and cannot be described by a sum of a few Lorentzians. The spectra clearly demonstrate a presence of two well separated relaxation processes, slow and fast.[15,17–19] Both are stretched (nonexponential), but the slow process is stretched from the high-frequency side while the fast process is stretched from the low-frequency side. This is typical for relaxation spectra of many glassforming liquids.

The only purpose of the fit in our case is the separation of the vibrational and relaxational contributions to the Raman spectra and qualitative analysis of their temperature variations. Thus the exact spectral shape of the quasielastic contribution is not crucial for our analysis. Moreover, it is known that high frequency $\nu > 3-5$ cm^{-1} (or short time, $t < 1$ ps) part of the relaxation spectra in polymers and glass-forming systems have single exponential-like behavior and becomes nonexponential at lower frequencies or longer relaxation times.[20,24] So, the Lorentzian shape should be a reasonable approximation for the quasielastic spectra at $\nu > 5$ cm^{-1}. Bearing this in mind, we fit the low frequency region of the spectra with the sum of a single Lorentzian for the QES part and a lognormal distribution for the boson peak,

$$I_n(\nu) = \frac{A\nu_0}{\nu_0^2 + \nu^2} + B \exp\left\{ - \frac{[\ln(\nu/\nu_{\mathrm{BP}})]^2}{2[\ln(W/\nu_{\mathrm{BP}})]^2} \right\}. \quad (1)$$

Here, ν_0 and A are the width and the intensity of the Lorentzian, ν_{BP} is the frequency, W is the width and B is the amplitude of the boson peak. The log-normal distribution is a traditional approximation for the asymmetric shape of the boson peak in disordered systems,[21–23] and it fits well the peak in lysozyme up to ~ 60 cm^{-1} (Fig. 7). Contributions of higher frequency modes become significant at higher ν. Previously we mentioned that the relaxation spectra of biopolymers are complex. However, here we can use a single Lorentzian because of the low frequency limit used in these measurements (≈ 3 cm^{-1}); one needs access to considerably lower wavenumbers to see the distribution of relaxation times.[17–19] Fitting the spectra with this single Lorentzian does not reveal any significant temperature dependence for ν_0. This is typical for disordered systems, as shown by Surovtsev and co-workers.[24] The typical fits are shown in

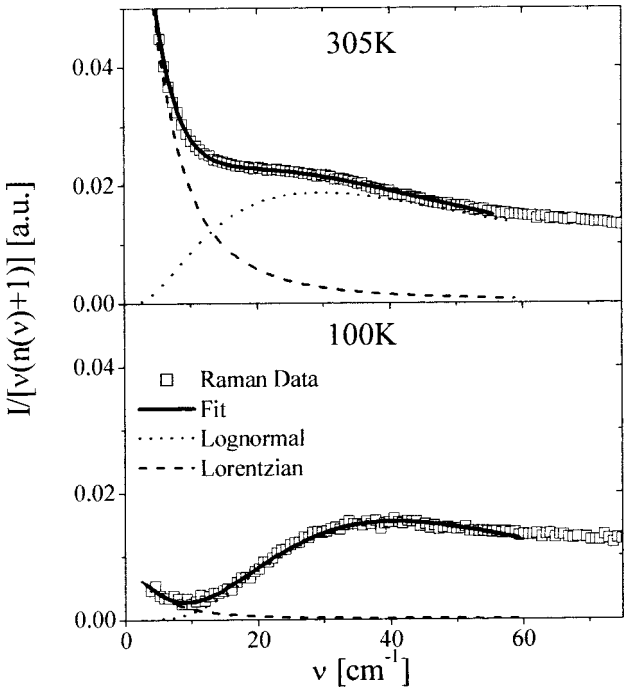

FIG. 7. Raman spectra of wet sample (symbols) and their fit using Eq. (1). Boson peak and QES contribution estimated from the fit are shown separately.

Fig. 7. This simple approximation provides reasonable fit of the Raman spectra for all the samples at all temperatures (shown as the solid lines in Fig. 4).

The resulting boson peak frequencies and integrated QES intensities are presented in Fig. 8. At low temperatures, the boson peak has the highest frequency in wet lysozyme and lysozyme dissolved in glycerol, but it softens sharply at temperatures above 200 K [Fig. 8(a)]. The boson peak in dry and lysozyme:trehalose samples has a significantly lower frequency at low T, but then it shows only mild variations with temperature [Fig. 8(a)]. The fit QES intensity variations [Fig. 8(b)] are similar to results from the direct integration of the I_n presented in Fig. 6. The notable exception is the difference between dry and lysozyme:trehalose samples. This is due to lower frequency of the boson peak in the dry state [Fig. 8(a)]. Part of the low frequency tail of the boson peak contributes in the 5–8 cm^{-1} window where the integration for Fig. 6 was performed.

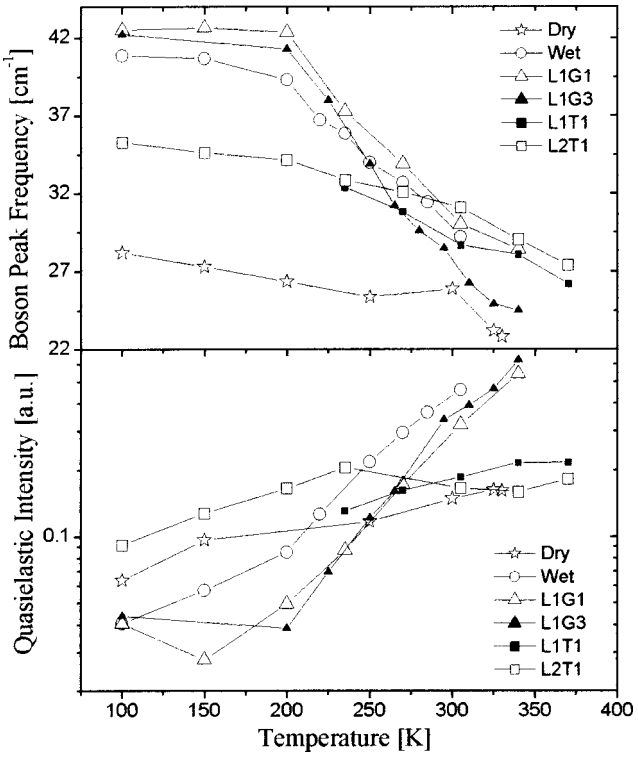

FIG. 8. Results of the fit for the frequency of the boson peak maximum ν_{BP} (a) and integrated quasielastic intensity A (b).

DISCUSSION

It is widely believed[17,25] that proteins demonstrate harmonic motion below their dynamic transition temperature T_d. The dynamic transition is usually defined as a sharp rise of mean squared atomic displacement $\langle x^2 \rangle$. Below the dynamic transition, $\langle x^2 \rangle$ increases nearly linearly with T, a characteristic of a harmonic solid. Hydrated proteins display T_ds around 200–230 K while dehydrated proteins typically do not exhibit a T_d.[17,25,26] Neutron scattering experiments indicate a T_d near 220 K for hydrated lysozyme, and around 270 K for lysozyme in glycerol, whereas no transition has been observed up to 430 K for the dry protein.[26]

Harmonic motions should give a temperature independent spectral density $I_n(\nu)$. This harmonic behavior is observed for the lysozyme samples only at high ($\nu > 75$ cm^{-1}) frequencies (Fig. 4). The low-frequency spectra reveal anharmonic motions for the lysozyme/glycerol and wet samples above 150–200 K, and over the entire temperature range for the lysozyme:trehalose and dry samples (Fig. 4). This anharmonicity shows up as an increase in the quasielastic intensity and as the shift of the boson peak with temperature (Figs. 4, 5–8). Similar anharmonicity was observed in the detailed analysis of neutron scattering spectra of dry and wet DNA.[18,27] This clearly contradicts the previous notion of purely harmonic motions below T_d.

The frequency of the boson peak appears to be very sensitive to environmental conditions [Fig. 8(b)]. It is the highest in solid lysozyme:glycerol sample at low T, decreases in lysozyme:trehalose sample and is the lowest in the dry state, i.e., with no solvent surrounding the protein. ν_{max} decreases slightly with an increase in temperature for both the dry and trehalose environments. We also notice a sudden drop of the boson peak frequency in water and glycerol environments above 170 to 200 K, which correlates with the sharp decrease of elastic constants of these solvents above their glass transition temperatures, T_g. These observations support the idea that the boson peak vibrations involve the entire protein molecule. The frequency of these global vibrations would be very sensitive to the elasticity of the surrounding solvent. This is consistent with the picture from recent molecular dynamic simulations[28] where it was shown that side chains and backbone, surface and inner part of a protein are all involved in the boson peak vibrations.

Let us now turn to the analysis of the QES. The QES intensity reflects internal relaxationlike motions of a protein,

i.e., local conformational transitions between substates at a picosecond time scale. These relatively fast, local relaxations are necessary precursors for the much slower, global protein motions requisite for biological activity. Analysis of the QES intensity provides a powerful tool to directly observe how a given preservation agent influences these precursor motions of the protein, without relying on mechanism dependent models or computer simulations.

At high temperatures it appears that water and glycerol impart greater flexibility to the protein, leading to stronger conformational fluctuations than observed in the dry lysozyme. Increased conformational flexibility in water and glycerol was observed in many different experiments and interpreted as ''lubrication'' by the solvent.[29,30] It is known that higher local conformational flexibility leads to lower thermal stability of proteins.[30] This is supported by a correlation of the quasielastic intensity [Figs. 6 and 8(b)] and the lysozyme melting temperature T_m obtained from differential scanning calorimetry (DSC) measurements.[31] According to the DSC measurements, T_m decreases from 430 K in the dry state to 370 K in L1G1, and to 340 K in the wet lysozyme.[31] The temperature shift in the rising QES intensities between the wet and L1G1 samples is also 30–40 K [Figs. 6 and 8(b)], i.e., of the order of the difference in T_m's of these two samples. Thus, the molecular motions responsible for the increase in the QES intensity reflect the thermal stability of the protein.

At low temperatures, glycerol appears to be the most effective solvent for suppressing the local motions of the protein. Water also strongly suppresses the conformational activity of a protein at low temperatures. This suppression far exceeds the simple cooling effect observed in dry lysozyme. In comparison, trehalose allows greater conformational fluctuations in the protein at low temperatures [Figs. 6 and 8(b)]. This unexpected result is already visible in Fig. 5. Observations of a higher QES intensity in dry vs wet samples at low temperatures have been reported in neutron scattering experiments on myoglobin,[14] α-amylase,[32] and DNA.[18,27] Thus, suppression of conformational motion by water of hydration at low temperatures is a general property for different biopolymers. The same seems to be true for glycerol [Figs. 6 and 8(b)].

The unexpected result that liquid glycerol suppresses the fast relaxations in lysozyme better than solid trehalose agrees with time-dependent geminate CO recombination measurements for myoglobin (Mb) in glycerol[33] and trehalose.[8,34] Near 200 K, the ligand escape and conformational rearrangements are faster in the Mb dissolved in trehalose glass as compared to Mb in glycerol.[8,33,34] This is consistent with the stronger low temperature suppression of the QES intensity induced by glycerol. Using a model-dependent analysis, it has been found[8] that activation energy for conformational rearrangements of Mb dissolved in glycerol is approximately 3 times higher than those in Mb dissolved in trehalose. Using another model approximation Hagen *et al.*[35] also found that activation barriers are 1.5–2 times higher in Mb dissolved in glycerol than those in Mb dissolved in trehalose. These observations are consistent with the milder temperature dependence of the QES in the lysozyme/trehalose samples [Figs. 6 and 8(b)]. It was shown[8] that above 270 K there is a crossover and the conformational diffusion and ligand escape become faster in the myoglobin/glycerol sample as compared to myoglobin/trehalose system. This is exactly analogous to the crossover in the protein conformational fluctuations observed in the QES intensities [Figs. 6 and 8(b)]; above 270 K fluctuations become greater in the lysozyme dissolved in glycerol.

The similarities between the CO rebinding kinetics in myoglobin and the integrated QES intensities in lysozyme for the trehalose and glycerol environments are striking and support a direct relation between the QES intensity and biological activity. Trehalose is very effective at preserving many proteins and living organisms against dehydration under ambient temperatures. On the other hand, glycerol, providing little suppression of dynamics (relative to the wet protein) under ambient conditions, is very effective at suppressing protein dynamics under cryogenic conditions.

There are also recent spectral diffusion experiments on horseradish peroxidase in trehalose and glycerol based buffers at very low temperatures.[36] These experiments lead to the same conclusion; trehalose increases the conformational flexibility of the protein relative to the glycerol/water buffer.[36] Thus, our interpretation based on Raman scattering is supported by multiple independent measurements.

The notion that trapping a protein in a highly viscous glass confers stability might suggest that a higher T_g glass is a superior preservation agent.[37] However, the T_gs of glycerol and trehalose are 193 and 387 K, respectively. Hence there is window between 193 and 270 K where the viscous liquid glycerol is more effective at suppressing the dynamics and activity than the glassy trehalose. Clearly this demonstrates that there is more to protein stabilization than glass formation alone. This points to arguments[38] that conformational change in a protein ''may not be frozen (with trivial temperature effects) so much as stuck'' under the influence of a solvent. One must consider not only the viscosity of the preservation agent, but also the interactions with the protein and any structural water that may be retained by the protein.

At present, there is no clear explanation why glycerol suppresses the protein dynamics more efficiently than trehalose at low temperatures, and vice versa at high temperatures. There are reports[39] that the escape rate of CO from Mb is faster in sucrose–water solutions than in glycerol–water solutions under conditions where the viscosities of the solvents are identical. The authors explained this finding using ideas of preferential hydration. It is well known that preferential hydration of proteins occurs in aqueous glycerol and trehalose solutions.[40,41] This effectively changes solution viscosity around the protein surface. The preferential hydration, however, cannot explain our observations [Figs. 6 and 8(b)] because at low temperatures suppression of dynamics in wet sample is stronger than that in lysozyme/trehalose sample. It is difficult to believe that viscosity at the surface of protein embedded in trehalose can be lower than in wet protein. That the suppression of internal protein motions can be greater in the liquidlike glycerol in comparison to glassy trehalose suggests that glycerol interacts more intimately with lysozyme. Glycerol is a relatively small molecule and it is feasible that

it can access and directly interact with the interior regions of the protein. On the contrary, trehalose is relatively bulky and the damping of the internal dynamics probably occurs through an indirect viscous coupling mechanism, acting through the periphery of the globular protein.[42–44] It has been shown that the specific volume of proteins (including lysozyme) decreases in glycerol,[29] which should then lead to a suppression of the conformational fluctuations. If the thermal expansion coefficient of liquid glycerol is larger than in solid trehalose, one could imagine that temperature changes might lead to much stronger changes of protein's specific volume in glycerol. Speculations like these may explain why trehalose is less effective than glycerol at suppressing protein dynamics at low temperatures.

CONCLUSIONS

The exact mechanisms by which glycerol and trehalose confer protein stability still remain unclear. Regardless, the presented above analysis demonstrates that observed variations of the QES intensity correlate with changes in biochemical activity and thermal stability of the protein in various environments. It is shown that the glass formation itself is not sufficient for effective suppression of protein dynamics. At high temperatures, trehalose provides superior dynamical suppression and stability. Conversely, liquid (as well as glassy) glycerol appears to impart to better stability than glassy trehalose at low temperatures. We suggest that these counterintuitive results might be due to difference in protein–solvent interactions. Regardless of the nature of these interactions, low frequency Raman scattering appears to be an easy and viable tool to evaluate the dynamical implications of protein stabilization schemes. However, we emphasize that low frequency Raman does not take into account potential adverse interactions between the protein and solvent, such as unfolding. That information can be obtained by analyzing the amide vibrations (high frequency range, 1200–1700 cm^{-1}) that are sensitive to hydrogen bonding inside the protein and its interaction with solvents.[45] We plan to compare these traditional high-frequency Raman measurements with our low-frequency spectra on the same samples.

ACKNOWLEDGMENTS

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