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Influence of solvent on dynamics and stability of a protein

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Abstract

Proteins are often dissolved in viscous glass-forming solvents to provide thermal stability and preserve biochemical activity. However, the mechanisms by which this preservation is achieved are unclear. This issue of biopreservation is undoubtedly affected by both thermodynamic and dynamic parameters. The latter parameters will control the rate of conformational transitions of the protein that accompany biological activity. In the present communication we observe variations of local conformational motions of lysozyme in different solvents by using low-frequency Raman spectroscopy. We demonstrate that at low temperatures liquid glycerol provides a stronger suppression of the fast conformational motions of the protein than glassy trehalose. This demonstrates that solvent viscosity is not the only parameter that controls protein dynamics, and details of the protein–solvent interactions might be important in the biopreservation process.

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1. Introduction

Understanding of protein–solvents interactions is extremely important for storage of protein-based pharmaceutical, cells, tissues and organs. It is well known that many organisms can survive extreme temperatures and/or dehydration by synthesizing significant amounts of some sugars or polyalcohols in both their intra- and intercellular fluids [1]. The compounds in these naturally occurring biopreservation processes, like glycerol and trehalose, are now widely used in pharmaceutical industry [2]. However, the mechanism of protein stabilization by these substances remains unclear. Many authors speculate that solvent viscosity plays a crucial role in suppressing dynamics within the protein and thereby decreasing the biochemical activity [3,4]. This idea has led to speculations [5] that a higher glass transition temperature (T_g) solvent is better for protein preservation. In particular, trehalose is considered to be one of the best solvents for biopreservation since it has the highest T_g among sugars [5].

Kinetics of CO or oxygen rebinding to myoglobin (Mb) or hemoglobin (Hb) is a traditional model process for analysis of kinetics of protein

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function and its dependence on temperature and solvent. Starting from pioneering work of Frauenfelder and coworkers [3,6], a large amount of experimental and theoretical works has demonstrated the influence of solvent viscosity on the kinetics of biochemical reactions [3,4,6–11]. It has been found, however, that the solvent viscosity is not the only parameter that controls the kinetics of a ligand rebinding [8,10,11]. For example, it was shown [11] that the same rate of chemical reaction occurs in sucrose and glycerol aqueous solutions that have viscosities differing by $\sim 10^7$ times. These observations stress the importance of protein–solvent interactions and coupling of internal protein dynamics to the solvent dynamics.

Raman and IR spectroscopy are often used to analyze protein–solvents interactions. Traditionally the high-frequency ($\nu \sim 1000$–1700 cm^{-1}) amide modes are studied because they are very sensitive to the secondary structures in a protein and the hydrogen bonding between solvent molecules and protein [12]. It has been also found that low-frequency ($\nu < 100$ cm^{-1}) Raman and neutron scattering spectra of proteins are extremely sensitive to degree of their hydration and temperature [13,14]. In present communication, we use low-frequency ($\nu < 100$ cm^{-1}) Raman scattering to directly probe internal conformational motions in the protein lysozyme dissolved in different solvents.

2. Experimental

Hen egg white lysozyme was obtained from Sigma [1] and used without further treatment. The as received powder was designated as the 'dry' sample. 'Wet' lysozyme was obtained by exposing the dry protein powder to 98% relative humidity, over a saturated potassium sulfate solution, at room temperature for approximately three weeks. The resulting moisture uptake was 35–40% by mass. The dry protein was also directly mixed into a 1–3 mass-ratio with glycerol, resulting in a homogeneous, optically transparent solution (L1G3). The L1G3 sample was placed in an optical ampoule and hermetically sealed. Stabilized aqueous solutions were created by dissolving equal mass fractions of dry lysozyme powder and glycerol (L1G1) as well as lysozyme:trehalose mixtures at mass ratios of 1:1 (L1T1) and 2:1 (L2T1). These aqueous solutions were freeze-dried into powders and maintained at sub-ambient temperatures until the time of measurement. All the powders were sealed between sapphire windows. Sapphire does not have a significant contribution to the Raman signal in the low-frequency region ($\nu < 100$ cm^{-1}) of interest.

The Raman scattering spectra were measured in the back-scattering geometry using a triple-monochromator Jobin Yvon T64000 with a 514.5 nm Ar^{2+} laser with 10–25 mW of power incident on the sample. The spectra were recorded with a multi-channel detector. Detailed analysis [15] shows that the spectra below 100 cm^{-1} are dominated by the signal from the protein and the solvents (glycerol, water and trehalose) give negligible contribution in this spectral region. Special care was taken to avoid the tail of the elastic line in low-frequency ($\nu < 10$ cm^{-1}) region.

The statistics in our Raman measurements are very high. The main source of error of the scattering intensity at low frequencies comes from a fluorescence background. As temperature decreases, this background increases while the Raman signal decreases. Our estimates show that the maximum possible error in the Raman intensity (due to correction for the fluorescence background) is $\sim \pm 20\%$ at $T = 100$ K (the lowest T of our measurements), and decreases sharply at 200 K and above. As will be seen later, this low temperature error is considerably less than the differences between the different samples. At least two separate samples were prepared for each environmental condition, with multiple measurements performed on each sample to ensure reproducibility; significant differences were not observed within the accuracy of our measurements.

[1] 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.

3. Results

Fig. 1 shows low-frequency Raman spectra in terms of spectral density, $I_n = I/[\nu(n(\nu)+1)]$, where the scattering intensity I is normalized 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 variations. In a purely harmonic system, Bose scaling results in temperature independent spectral densities. However, such harmonic behavior is observed only at frequencies higher than 75 cm^{-1} and the spectra demonstrate significant temperature variations at lower ν.

The low-frequency Raman spectra contain two primary components [13,16,17]: (i) an 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}. For proteins, the former corresponds to low-frequency, collective amino-acid vibrations related to the elasticity of the protein. QES reflects local conformational fluctuations and higher QES intensity corresponds to greater protein flexibility and faster local rearrangements of amino acids.

In Fig. 1, contribution both from the Boson peak and the QES are clearly visible. The main variations are (i) an increase in the QES intensity and (ii) softening of the Boson peak with increasing temperature. In this short communication, we restrict our discussion to QES, which reflects the fast relaxation. Both variations are strong in wet lysozyme and lysozyme/glycerol sample 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 a difference of only a factor of two is observed in the dry protein and the protein preserved in trehalose.

Fig. 2 shows the spectra for two temperatures, 200 and 305 K. The QES intensity is low for wet and glycerol dissolved protein samples at 200 K while it is relatively high for dry and trehalose

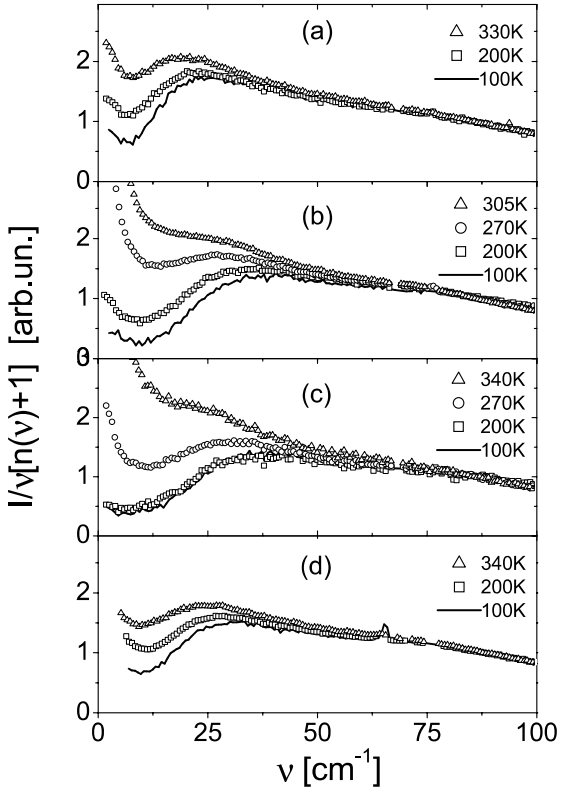

Fig. 1. The low-frequency Raman spectra of dry lysozyme (a), wet lysozyme (b), lysozyme/glycerol sample (L1G1) (c) and lysozyme/trehalose sample (L2T1) (d) at different temperatures.

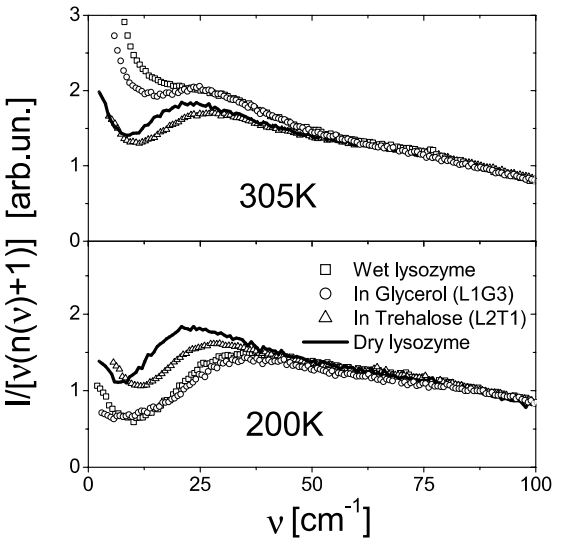

Fig. 2. The low-frequency Raman spectra of lysozyme in different solvents at two selected temperatures. The intensities are normalized at frequencies $\nu \sim 75$–150 cm^{-1}.

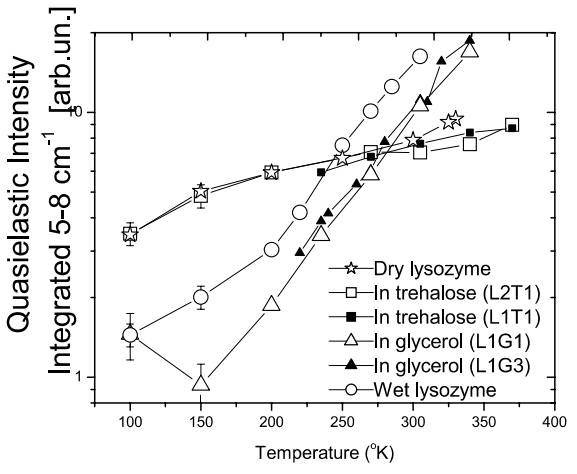

Fig. 3. Normalized quasielastic intensity $I_n(v)$ integrated over the frequency range 5–8 cm^{-1} for different lysozyme samples. Error-bars are shown at low temperatures only. They decrease with increase in temperature.

embedded proteins. At 305 K, we see the reverse situation; the wet and glycerol coated samples have higher QES intensity than the dry and trehalose embedded protein. Fig. 3 presents temperature variations of the QES intensity integrated over $v = 5$–8 cm^{-1}. These data are presented in a logarithmic scale in order to emphasize stronger the peculiarities at the low temperature side (low intensity region). For low temperatures ($T < 200$ K), both water and glycerol substantially suppress the conformational jumps in lysozyme, more so than either the trehalose containing or dry states. However, a rapid increase in the QES intensity occurs near 150–200 K for the wet and glycerol coated samples. The QES intensities in wet and lysozyme/glycerol samples exceed that of the dry lysozyme near 250 and 280 K, respectively. An important point to notice in Fig. 3 is that the dynamics of trehalose-containing sample follow those of the dry lysozyme. Both samples show a subtle QES variation with temperature in comparison to lysozyme in either the glycerol or water. Agreement between the samples with different contents of a protein in the solvents (either glycerol, L1G1 and L1G3, or trehalose, L1T1 and L2T1) suggests that solvent concentration is not a determining factor in this range.

4. Discussion

The quasielastic light or neutron scattering spectra reflect internal relaxation-like motions of proteins. These motions are conformational transitions between substates that occur on a time scale of pico- to nanoseconds. It is believed that these conformational motions are the necessary precursors for the biochemical activities of proteins [18].

The QES intensity of the lysozyme displays similar thermal variations above 200 K in both the hydrated and glycerol environments, with only an apparent temperature axis shift of ~30–40 K for the glycerol sample (Fig. 3). It is well known from calorimetry [19] that the melting temperature T_m of dry lysozyme is about 430 K and drops to 370 K in the presence of glycerol, and to 340 K in hydrated state. The shift in the integrated QES intensities from our Raman measurements is in quantitative agreement with the 30 K difference in T_ms.

At room temperature, Fig. 2 suggests that the lysozyme in both the humid and glycerol environments has higher intensity of conformational fluctuations than either the dry or trehalose conditions. Phenomenologically this might be explained by a plasticization effect [20]. It is well known that lysozyme in glycerol will fold into secondary and tertiary structures that are very similar to those formed in water [21,22]. From the crystal structure of lysozyme, we know that water resides both at the hydrophilic periphery of the proteins as well as deep inside the folded structure. Taken together, these facts suggest it is likely that glycerol will also penetrate into the core of the protein lysozyme; it is a relatively small molecule that could help satisfy some of the hydrogen bonds provided by the structural water. These small molecules, whether they are glycerol or water, act as molecular level 'lubricants' at ambient conditions where they themselves have sufficient mobility. This could possibly explain the high degree of mobility in the wet and glycerol coated lysozyme under ambient conditions.

We do not see the conformational mobility enhancement effect in the case of trehalose. Trehalose being a much bigger molecule in comparison to glycerol, probably experiences geometric or

steric constraints to penetrate through the folded amino acid chains. Since this sample has been lyophilized, there should not be enough water molecules to act like lubricants. Thus, most probably, trehalose encapsulates lyophilized protein without significant influence on its dynamics. This would explain why behavior of the protein in trehalose follows that of the dry sample.

This picture is consistent with mean-squared H-atoms displacements $\langle x^2 \rangle$ measurements by neutron scattering on Mb [23]; $\langle x^2 \rangle$ in both dry and trehalose environments displays a mild temperature variation, while wet Mb shows much larger $\langle x^2 \rangle$ above $\sim$220 K, i.e. above the dynamic transition. Recent neutron measurements on lysozyme [24] also demonstrate that $\langle x^2 \rangle$ at ambient temperature is the highest for the wet sample, lower for L1G1 sample (due to shift of the dynamic transition) and is the lowest for the dry protein.

At low temperatures, however, the situation changes in Fig. 3; the wet and lysozyme/glycerol samples have lower QES intensities than the dry or trehalose coated samples. Weaker QES intensity in the wet biopolymers as compared to their dry state at low temperatures has been observed earlier in neutron scattering experiments for Mb [16], α-amylase [25] and DNA [26]. Thus, this result seems to be well established. To the best of our knowledge, no similar measurements with trehalose coated samples were published. The main problem here is that contribution from trehalose dominates the neutron scattering spectrum. In that respect, our data provide a more complete picture with broader variety of solvents. The most important result (Fig. 3) is that at low temperatures liquid glycerol suppresses dynamics of lysozyme stronger than solid trehalose. Small molecule plasticizers often have an opposite effect and lead to a loss of mobility. When these small molecules are frozen into the macromolecular structure, their steric interactions reduce the degrees of freedom and increase the rigidity of the structure. Typically this is understood as a filling in of the empty regions between chains and thus an increase the packing efficiency. Fig. 3 is consistent with this antiplasticization picture at low temperatures. The dynamics are severely curtailed below 270 and 240K in the glycerol containing and wet lysozyme, respectively. This is in contrast to both the dry and trehalose containing samples that do not show the antiplasticization effect and consequently have greater conformational mobility at low temperatures.

It is worthwhile to compare our observations to biochemical activity measurements of proteins in glycerol and trehalose. While no such data are readily available for lysozyme in the literature, they are available for Mb and Hb. One can measure the Hb– or Mb–CO recombination rates, which directly reflects the biochemical activity of these proteins. The rebinding kinetics for Mb or Hb in aqueous solutions of sugars and glycerol has been compared in many different papers [7–11]. It has been demonstrated that the kinetics reach similar rate when viscosity of sucrose aqueous solution is $\sim 10^3$–10^7 times higher than viscosity of glycerol aqueous solution [10,11]. Analysis presented in [7] clearly demonstrates that the fast rebinding process at room temperature is rather similar for Hb dissolved in glycerol and in trehalose, although they have very different viscosities. In particular, using rather complicated model analysis, Sastry and Agmon conclude [8] that near 200 K, the ligand escape and conformational rearrangements are faster for Mb dissolved in trehalose as compared to Mb in glycerol. This is consistent with the stronger suppression of the QES intensity induced by glycerol at low T. Using this model, the authors of [8] estimated that the activation energy for conformational rearrangements of Mb in glycerol is approximately three times higher compared to Mb in trehalose. Using another model approximation, the authors of [9] also found that activation barriers are higher (but only $\sim$1.5–2 times) in Mb dissolved in glycerol than in Mb dissolved in trehalose. This is consistent with the milder temperature dependence of the QES intensity in the lysozyme/trehalose samples (Fig. 3). It was also shown in [8] that above 270 K there is a crossover and the conformational diffusion and ligand escape processes become faster in the Mb/glycerol sample compared to Mb/trehalose system. This is exactly analogous to the crossover in the protein conformational fluctuations observed in low-frequency Raman: above 270 K fluctuations become larger in the glycerol preserved

lysozyme. We would like to emphasize that the analysis presented in [8] is strongly model dependent. Thus one should not overestimate the quantitative agreement of the crossover temperatures obtained from our Raman data and from CO rebinding kinetics. Regardless, all measurements on ligand rebinding [8–11] demonstrated that proteins dissolved in sugars have higher conformational activities than proteins in glycerol at the same viscosity of the solvents. Whether it can be explained by 'preferential hydration' (as it is suggested in [11]), by decoupling of protein and solvent dynamics (proposed in [8]) or by another mechanism remains unclear.

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

The results demonstrate one very important fact: A higher viscosity or higher T_g solvent does not always lead to better biopreservation. Between 193 and 270 K, the liquid glycerol provides greater stability than the glassy trehalose. There is a window of at least 70 K where a liquid solvent suppresses dynamics and biochemical activities better than a solid one. This agrees with the previous speculations of Ansari et al. [4] suggesting that the conformational changes in a protein 'may not be frozen (by a trivial temperature effect) so much as stuck (under the influence of a solvent)'. Our observations also support speculations proposed in [8] that internal dynamics of proteins dissolved in trehalose is decoupled from the solvent dynamics, at least, at low temperatures. That explains similarity of the spectra of the dry sample and lysozyme/trehalose samples at low temperatures (Figs. 2 and 3).

The exact mechanisms by which glycerol and trehalose confer protein stability still remain unclear. We have discussed one possible explanation in terms of a plasticization–antiplasticization effect. Regardless, the presented analysis demonstrates that observed variations of the QES intensity correlate with changes in biochemical activity and thermal stability of the protein in various environments. We have shown that the glass formation itself is not sufficient to guarantee maximal suppression of protein dynamics. At low temperatures, liquid (as well as glassy) glycerol appears to impart to better suppression of protein dynamics than glassy trehalose. Conversely, trehalose provides superior dynamical suppression and stability at higher temperatures. We suggest that one of the most important factors is the interaction of the solvent with the biomolecule. A high interacting agent may influence the conformational mobility stronger than normal cooling effect. The local conformational jumps dynamically constrained by interactions with the solvent molecules result in slowing down of the bioactivity and denaturation process.

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