

Inertial Suppression of Protein Dynamics in a Binary Glycerol–Trehalose Glass

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The traditional approach used to predict the ability of a glassy matrix to maximally preserve the activity of a protein solute is the glass transition temperature (T_g) of the glass. Recently it has been shown that the addition of a low T_g diluent (glycerol) can rigidify the structure of a high T_g glassy matrix in binary glycerol–trehalose glasses. The optimal density of glycerol in trehalose minimizes the average mean square displacements of non-exchangeable protons in the glass samples. The amount of glycerol added to a trehalose glass coincides with the maximal recovery of biological activity in a separate study using similar binary glass samples. In this study, we use molecular dynamics (MD) simulations to investigate the dynamics of a hydrated protein encased in glycerol, unary trehalose and binary glycerol–trehalose glasses. We have found that we are able to reproduce the rigidification of the glycerol–trehalose glassy matrix and that there is a direct correlation between bulk glass dynamics and the extent of atomic fluctuation of protein atoms. The detailed microscopic picture that emerges is that protein dynamics are suppressed mainly by inertia of the bulk glass and to a lesser extent specific interactions at the protein–solvent interface. Thus, the inertia of the glassy matrix may be an influential factor in the determination of pharmaceutically relevant formulations.

The use of glasses of disaccharides to maintain the biological activity of proteins has largely been motivated by the observation that many natural living systems utilize carbohydrates to survive severe environmental conditions such as low temperature or moisture content.¹ Of the disaccharides, α,α -trehalose is utilized in nature² and is generally thought of as having the best biopreservative abilities in vitro.³ Trehalose has been studied extensively in both liquid and amorphous phases by a variety of experimental^{4,5,7,9,14} and theoretical^{8,9,15} methods. There are many theories that have been advanced to account for the unique preservative properties of trehalose. Mechanisms that are applied to the amorphous state include those that invoke either the inclusion⁴ or exclusion⁵ of water from the surface of proteins, direct interaction of trehalose by anchoring to the protein,⁶ and intrinsic viscosity effects of the glass alone.⁷ The effect of the latter mechanism has been ascribed to the high T_g of trehalose⁸ and therefore it is claimed to be responsible for the preservation of functional proteins in the vitreous state. Unfortunately, these mechanisms generally do not account for the various observed kinetic and dynamic properties of such systems^{3,9} and thus it has been difficult to extend these ideas to other molecular biological preservatives.^{3,10} Thus, the traditional approach used to predict the ability of a glassy matrix to maximally preserve a protein solute is the glass transition temperature (T_g) of the glass. Indeed, it has been suggested that the higher the T_g the better preservation capabilities of the glass.⁸

Recently it has been shown that the addition of a low T_g diluent (glycerol) can rigidify the structure of a high T_g glassy matrix in binary glycerol–trehalose glasses.^{11–13} The concentration dependence of the dynamics, as determined by incoherent neutron scattering, indicates that there is an optimal amount of glycerol required to rigidify the binary glass system at high temperatures. The optimal density of glycerol in trehalose minimizes the average mean square displacements, $\langle x^2 \rangle$, of non-exchangeable protons in the glass samples. The amount of glycerol added to a trehalose glass coincides with the maximal recovery of biological activity in a separate study using similar binary glass samples.¹² Therefore it has been inferred that the rigidification of the bulk glass matrix is responsible for the long-term preservation qualities of these binary glasses.

Our goal is to develop validated computational methods to determine the microscopic factors of protein preservation in glasses. Typically, both experimental¹⁴ and theoretical¹⁵ investigations of proteins in trehalose or glycerol have focused on unary compositions. In this study, we use molecular dynamics (MD) simulations to investigate the dynamics of a hydrated protein encased in glycerol, unary trehalose, and binary glycerol–trehalose glasses. We have found that we are able to reproduce the rigidification of the glycerol–trehalose glassy matrix, and that there is a direct correlation between bulk glass dynamics and the extent of atomic fluctuation of protein atoms. The detailed microscopic picture that emerges is that protein dynamics are suppressed mainly by the inertia of the bulk glass, and to a lesser extent specific interactions at the protein–solvent interface.

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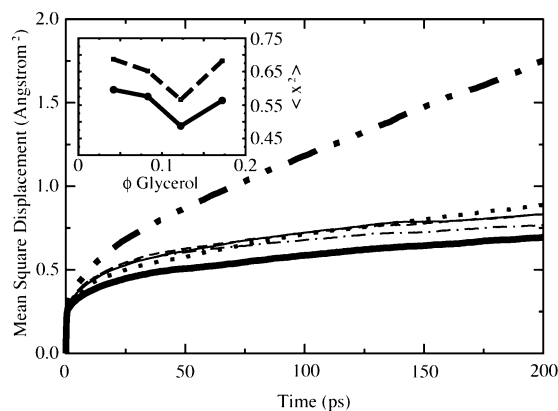


Figure 1. Mean square displacement, $\langle x^2 \rangle$, of non-exchangeable protons in the protein RNase as a function of time at 325 K for the glassy matrices: trehalose (thin solid line), 4.2% glycerol (dash line), 8.3% glycerol (dot dash), 12.2% glycerol (thick solid line), 17.2% glycerol (dotted line), 89% glycerol (double dot/dashed line). Inset: $\langle x^2 \rangle$ for non-exchangeable protons in RNase (solid line) and trehalose (dashed line) molecules as a function of glycerol content. Values are corrected for overall translation and rotation.

The results reported here were derived from 10 ns MD trajectories of the protein ribonuclease A (RNase) in hydrated amorphous periodic boxes at 325 K. Initial boxes used to encapsulate the protein were constructed from coordinates from a previous simulation study of dehydrated glycerol, trehalose, and glycerol–trehalose glasses.^{13,16} Our previous simulations have quantitatively predicted the picosecond dynamical behavior of proteins embedded in glasses by direct comparison of computed incoherent neutron spectra to recently available spectroscopic measurements.¹³ Five trehalose systems were constructed with various glycerol contents (0%, 4.2%, 8.3%, 12.2%, 17.2%) as well as a single glycerol system (89%). All percentages are reported by mass. Each system had a single RNase molecule ($\sim 6.5\%$), $\sim 4\%$ TIP3P water,¹⁷ and $\sim 0.1\%$ Cl^- ions. Initial protein–water configurations were taken from a 1 ns solution simulation where water molecules within 4 Å of the protein surface were retained. Subsequently, the protein–water cluster was transferred to the various glass systems by removal of overlapping trehalose or glycerol molecules. Simulations of the $\sim 28\,000$ atom systems were performed in the NPT ensemble using the CHARMM-22 force field¹⁸ utilizing the program NAMD.¹⁹ The final 2 ns of configuration data for each trajectory were used for analysis. The RMSD for protein backbone atoms for the various systems was < 2 Å compared to the starting solution structure. The average displacement of water molecules over the entire 10 ns trajectory was ~ 14 Å.

Figure 1 presents the mean square displacement, $\langle x^2 \rangle$, for non-exchangeable protons of RNase in the various systems at 325 K. This temperature was chosen since it is close to the predicted Kauzmann temperature of amorphous trehalose.⁹ The dynamics are most strongly suppressed in the 12.2% glycerol–trehalose glass at the concentration that has the maximum number of trehalose–glycerol hydrogen bonds as observed previously for protein-free simulations.¹³

A comparison of the $\langle x^2 \rangle$ of protein protons to trehalose protons as a function of glycerol content is shown in the inset of Figure 1. Clearly the dynamics of the protein follows that of the average dynamics of the trehalose molecules in the glass. The trend of $\langle x^2 \rangle$ versus glycerol content is similar to that found experimentally for dehydrated glycerol–trehalose protein-free glasses.^{11,13} The mole fraction of glycerol corresponding to maximum rigidity occurs at a higher glycerol content in our study which has a high concentration of protein (~ 7 mM). The

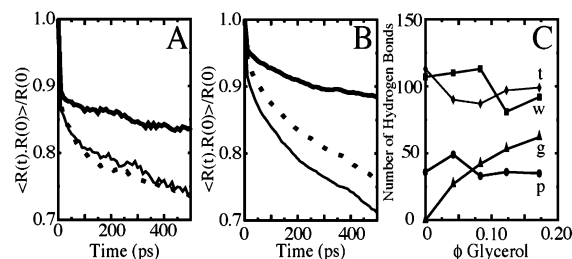


Figure 2. Residence time-correlation functions for (A) glycerol, and (B) water for a 4 Å shell from the surface of RNase A as a function of glycerol content: 4.2% glycerol (solid thin line), 12.2% glycerol (thick solid line), and 17.2% glycerol (dotted line). (C) Average protein hydrogen bond enumeration for protein–protein (p: circles), protein–water (w: squares), protein–trehalose (t: diamonds), and protein–glycerol (g: triangles) versus glycerol content (standard deviation of data in (C) $\sim \pm 10\%$).

effect on protein content on the amount of glycerol required to maximize the recovery of biological activity in binary glasses has been observed experimentally^{12b} and will be discussed further below.

To characterize the molecular determinants that lead to the suppression of protein dynamics in the various systems we have calculated the residence times of glycerol and water molecules about the protein surface as shown in Figure 2A and 2B, respectively. Here we define residence correlation functions, $\langle R(t) \cdot R(0) \rangle / R(0)$, for glycerol carbon atoms or water oxygen atoms. $R(t)$ was judged to be either 1 or 0 when the atom in question was within 4 Å (1) or greater than 4 Å (0) of the protein surface at the particular time t . On average, the glycerol and water molecules close to the protein surface stay in the vicinity longer in the 12.2% glycerol system compared to the other binary glycerol–trehalose glasses.

Hydrogen bond lifetimes, calculated as described previously,²⁰ did not reveal any significant differences in protein–solvent hydrogen bond durations between the various binary glasses. While both water and glycerol were found to remain near the protein surface longer in the case when the glass and protein were the most rigid, the hydrogen bond lifetimes were not distinctly different as a function of glycerol content. This may be a result of suboptimal sampling of non-equilibrium glassy systems or that the energetically favored packing arrangement at the protein–solvent interface is merely a balance of hydrogen bond strength and dispersive interactions as seen in amorphous disaccharide systems.²¹

Analysis of the number of protein–solvent hydrogen bonds indicates that there may be a preference for protein–trehalose hydrogen bonds over protein–water hydrogen bonds in the situation where the protein and glass dynamics is maximally suppressed as shown in Figure 2C. This result is consistent with the idea that protein preservation is dictated by the exclusion of plasticizing water from the protein surface by trehalose. However, the magnitude of the contribution of hydrogen bonding to the overall preservation of the protein is not known, especially in relation to the complete energetics of the system.

In Figure 3 we present various electrostatic and vdW pairwise interaction energies that were obtained by averaging the energies over the trajectories of the independent systems. As was found for pure glycerol–trehalose glasses,¹³ the maximum rigidification of the glass is found to correspond to the minimum energy composition of glycerol–trehalose interactions. We find that the sum of protein–solvent energetics follows that of the glass (Figure 3B,C) and that the individual contributions between glycerol, trehalose, and water are both more attractive (protein–

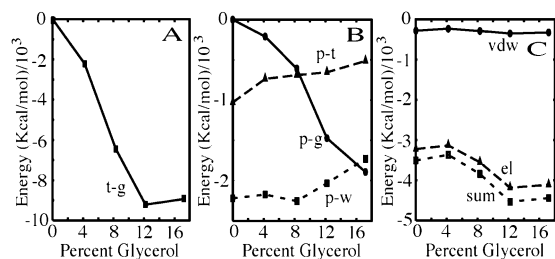


Figure 3. Pairwise electrostatic interaction energies for (A) trehalose–glycerol molecules (boxes), (B) protein–solvent molecules (protein–trehalose: p–t, triangles; protein–glycerol: p–g, circles; protein–water: p–w, squares), and (C) the sum of pairwise protein–solvent interaction energies as a function of glycerol content. In panel (C), vdW (circles), electrostatic (triangles), and total (squares) energies are shown (cal = 4.184 J).

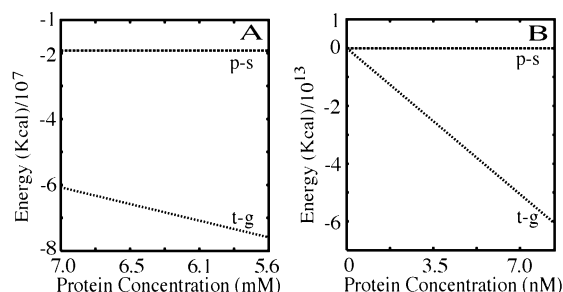


Figure 4. Total interaction energy as a function of protein concentration for protein–solvent (p–s: dashed line) and trehalose–glycerol (t–g: dotted line).

glycerol) and less attractive (protein–trehalose and protein–water) with increasing glycerol content (Figure 3B).

For the 12.2% glycerol binary glass system, the total number of solvent–solvent pairwise interactions is approximately seven times the number of protein–solvent pairwise interactions. In addition, as seen above, the magnitude of the normalized pairwise interaction energy of the glycerol–trehalose is twice that of the sum of protein–solvent energies. Thus, while the protein–solvent energetics follows the energetics of the solvent the system as a whole, it is largely influenced by the energetics of the bulk glass. One reason the amount of glycerol that is required to maximally rigidify the system is higher with increasing protein content (seen here and by experiment^{12b}) is that a certain amount of glycerol is required to bridge the bulk glass with the protein.

In Figure 4, we plot the energy for a binary glycerol–trehalose glass as a function of protein concentration. Values for trehalose–glycerol energies were estimated by extrapolation of the results obtained from the 7 mM simulation study using the energetics of a sub-volume of trehalose–glycerol molecules removed from the protein surface (e.g., energetics per mole of pairwise trehalose–glycerol interactions multiplied by the mole of interactions in the sub-volume). Clearly, the presence of a protein is a perturbation to the glassy matrix under the conditions of this study (7 mM), but the protein–solvent energy is less than 10^{-6} times than the glycerol–trehalose interaction energy at 7 nM. Thus, under the experimental conditions where maximal recovery of protein function upon reconstitution from a binary glycerol–trehalose glass (7 nM protein), the energetics of the glass far outweigh the energetics of the protein–solvent interface. Although, it is obvious that one should strive to obtain a rigid glass using compositions that do not disturb the native protein structure, which seems to be the case for glycerol–trehalose.

Finally, in Figure 5 we present the mean square fluctuations of both backbone and side chain heavy atoms of RNase for the

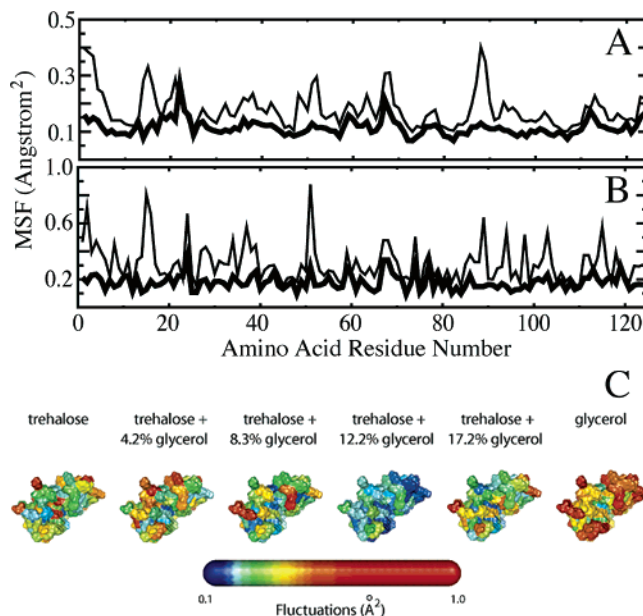


Figure 5. Mean squared fluctuations of heavy atoms in RNase A for (A) backbone atoms, and (B) side chain atoms for the 4.2% glycerol (thin line) and 12.2% glycerol (thick solid line) systems. The data are presented as the average over 100 ps time windows. In panel (C) a model of RNase A is shown with atoms colored by MSF for the different systems described in the text.

4.2% glycerol and 12.2% glycerol binary glass systems. The suppression of dynamical amplitudes at 12.2% glycerol versus 4.4% glycerol is almost completely uniform over the entire protein and is depicted graphically in Figure 5c.

In summary, our study suggests that the microscopic factors that lead to the maximum suppression of protein dynamics are primarily dictated by the inertial effects of the bulk glass rather than specific protein–solvent hydrogen bond interactions. The dynamics are suppressed throughout the protein, and this could have important consequences in the design of protein formulations. Thus, in the context of the current work, a new approach to optimize candidate pharmaceutical protein formulations would be to screen glassy systems for maximum rigidity while maintaining a degree of hydrogen bonding capability between the protein and the solvent, weighting rigidity over the hydrogen bonding.

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