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# Physical Insights into the Bio-preservation of Proteins by Glassy Solvents: Why is Glycerol better than Trehalose at low Temperatures?

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#### Abstract

Biopreservation has been a critical area of technical and scientific research as it enables various forms of biomolecular therapeutic agents to find practical use in medicine. The mechanism at which biomimicry-inspired solutions to stabilize biomolecules has been of great scientific interest. We have studied the behavior of lysozyme immersed in glycerol and trehalose, two solvents frequently used in the bio-preservation of proteins, with the purpose of identifying the microscopic origins of their very different dynamical suppression capabilities. In agreement with experiments, we find that glycerol is superior to trehalose at low temperatures, although the latter is deeper in the glassy state, while trehalose is better at higher temperatures. We traced the basis of this phenomenon to the different temperature dependencies of the intermolecular hydrogen bonds between the model protein structure and the surrounding solvent.

Keywords: structural dynamics, protein, solvent, biopreservation

## 1. INTRODUCTION

Biomolecules are essential for life. They represent therapeutic agents and targets in treating various medical diseases and conditions. Biological activity and properties of biomolecules (e.g., deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins) depend on their threedimensional (tertiary) structure. The tertiary conformational structure also dictates the biological activity of proteins such as enzymes, transporters, growth factors, receptors, antibodies, and signaling molecules. When biomolecules are introduced into environments with high or low temperature, acidity, pressure etc., they can go through significant and irreversible conformational changes (i.e., denaturation) that make them biologically inactive.

Understanding the molecular basis of the denaturation process and the investigation of the stability of proteins is an active area of interest in biological physics[1], [2]. Such an understanding is crucial in the development of various biomedical applications.[3] In fact, the scientific knowledge in this field built over the years has resulted in many real-life applications such as in developing detection kits for antibiotics resistant bacteria[4], lyophilized preservation of bone morphogenetic protein[5], biopreservation systems to preserve not just proteins but also cells[6], and stabilization of lyophilized antibody powders[7]. It is now well established that many sugars and polyols act as cryoprotectants in various species[3]. Hence, they are used in pharmaceutical applications of proteins as active ingredients. Their addition to the formulation help improve the stability of the active protein and extend the shelf life.

Among the various bio-preserving agents available, glycerol (1,2,3-propantriol) has proven to be very efficient in stabilizing the native state of proteins and the activity of enzymes at low temperatures.[8] Specifically, for lowtemperature applications, glycerol has proven to be superior to other bio-preserving agents such as sugars. However, one trehalose sugar, (α-D-glucopyranosyl α-Dglucopyranoside), is known to be more effective than glycerol at room temperature and it has been reported to be the most effective bio-protecting agent by means of functional recovery[9]. On the other hand, studies of geminate CO combination to myoglobin in trehalose and glycerol environments showed significant differences in terms of reaction kinetics.[10], [11] Counterintuitively, the rebinding kinetics was found to be faster in trehalose than in glycerol at low temperatures (<215 K). The mobility of myoglobin was higher in trehalose than in glycerol. This experimental finding has posed an unanswered question of how glycerol is more effective than trehalose at low temperatures.

The biological function of proteins shown to be reliant on structural fluctuations among their various conformational sub-states[12]. Consequently, the study of the dynamics of proteins is valuable for the understanding of the biological

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activity and the stabilization by the bio-preserving agents. Low-temperature Raman spectroscopy of lysozyme embedded in glycerol and trehalose has revealed that the former is a better bio-preserving agent than the latter even though trehalose is deeper in the glassy state.[13] On the other hand, trehalose was found to be more efficient at high temperatures. These results correlate with the kinetics of CO rebinding to myoglobin.[11] Namely, at low temperatures (< 200 K), glycerol inhibits the reaction kinetics more effectively. Caliskan et al.[13] argued that these counterintuitive findings in the dynamics of the protein are not due to the decoupling of protein-solvent interactions, rather the fragile character of trehalose. Namely, a lower energy barrier for conformational fluctuations in trehalose lead to a lower energy barrier for conformational fluctuations in the protein. However, experimental or theoretical evidence has not been presented and the nature of these interactions remains to be explored.

The main goal of this study is to identify and study the protein-solvent interactions based on the same model system (i.e., lysozyme in glycerol and trehalose) and to bring insights into to the peculiar influence of these two solvents on the dynamical behavior of proteins[14], [15] using Molecular Dynamics (MD) simulations.

#### 2. MODELING AND SIMULATION METHOD

The simulations were performed AMBER moleculardynamics package[16] with ff99 force field[17] to model lysozyme, trehalose and glycerol. The crystal structures of the solvent molecules (trehalose and glycerol) were optimized using the software package GAUSSIAN 03.[18] The same package was used to determine the electrostatic potentials on atom surfaces. The source of the 3-dimensional structure of our model protein (lysozyme, 193L) was retrieved a structural database.

The 6-12 Lennard-Jones potential was used for the van der Waals interactions. The Lennard-Jones parameters for the lysozyme-solvent interactions were calculated using the standard combination equations. The electrostatic interactions were determined using the particle-mesh Ewald method with a cutoff distance of 0.8 nanometers.

The initial velocities of atoms were assigned randomly using the Leap-frog Verlet algorithm. 1 fs of the simulation step size was used.

Rectangular and parallelepiped simulation box was created and periodic boundary conditions were used. Solvents were equilibrated at room temperature. The protein was placed in the middle of the simulation box after energy minimization.

The protein-solvent mixtures went through a series of equilibration steps in both the constant volume (first at 500 K and then at 300K) and the constant pressure conditions (0.1 MPa) for over 600 ps. After the equilibrium stages, the simulation was run at constant pressure conditions for over 2 nanoseconds.

The system was cooled down from an equilibrated state to low temperatures using 0.1 K/ps cooling rate for a 50K interval. Once the desired temperature has been reached, a further constant pressure equilibration simulation was run for 300 ps. Only after the system has reached the equilibrium, the data collection simulations were run.

### 3. RESULTS AND DISCUSSION

In order to reveal the impact of solvents on the dynamics of our model protein, i.e., lysozyme, within a nanosecond (ns) time range, we compared the dynamics of lysozyme in each solvent. We compared the ratio of the mean square displacement ( $\langle u^2 \rangle$ ) of lysozyme in glycerol to the one in trehalose at 1 ns resolution shown in Figure 1. Clearly, glycerol provides a better suppression of the dynamics as compared to trehalose at low temperatures in alignment with experimental studies[13].

We used incoherent intermediate scattering function (S(q,t)) in addition to  $\langle u^2 \rangle$  to study lysozyme's dynamics. The formula for S(q,t) is given below.

$$S(q,t) = \frac{1}{N} \left\langle \sum_{j=1}^{N} e^{i\mathbf{q}\cdot\mathbf{f}\left[j(t)\mathbf{R}_{-j}(0)\right]} \right\rangle \qquad (\text{Equation 1})$$

Here, **q** is the scattering wave vector, *t* is time in n. N stands for the number of hydrogen atoms,  $\mathbf{R}_{j}(t)$  is the three dimensional location of the *j*-th atom at time *t*. The brackets mean that the averaging takes place over time. This function presents important insights about the dynamic behavior and the relaxation of the biomolecule. Namely, since this function is presented over time scale, one can infer the various forms of relaxation from this representation such vibrations, conformational changes and translations.



**Figure 1.** The ratio of  $\langle u^2 \rangle$  for lysozyme hydrogen atoms in solvents at 5 different temperatures. Averaging of  $\langle u^2 \rangle$ (t) was carried out over 1 ns.

It is clear that in Figure 2a (as also in Figure 1), at elevated temperatures (i.e., 300 K), the pico-nanosecond range

dynamics of lysozyme in glycerol is faster than in trehalose. The physical origin of this result is intuitively explained by the fact that the liquid nature of glycerol and the glassy behavior of trehalose at this temperature. Indeed, glycerol is well above its glass transition temperature (Tg) of 192 K, while trehalose is well below its Tg of 388 K. At this temperature, glycerol allows fluctuations among the conformational sub-states of the protein beyond the harmonic-like motions,[14] whereas trehalose prevents such motions and limits the dynamics to harmonic-like motions.[15] However, at lower temperatures the dynamic behavior of lysozyme reverses. Indeed, at 200 K lysozyme becomes slower in glycerol than in trehalose, although the latter is substantially deeper in the glassy state. This result agrees with experiments performed on the same systems and was attributed to the superior effectiveness of glycerol in the preservation of biological agents at low temperatures[13].



**Figure 2ç** Figure 2a. S(q, t) of lysozyme in trehalose (continuous lines) and glycerol (dashed lines) at three temperatures. b. HB correlation function for the hydrogen bonds between the protein and glycerol (dashed lines) and trehalose (continuous lines) at three temperatures.

While the conformational denaturation of proteins occurs at a time scale which is substantially longer than the resolution of the experimental measurements and the current simulation study, the protein motions at this shorter time scale could be precursors to larger scale conformational changes.[19] Firstly, it is known that the rapid and small scale atomic fluctuations in the native conformation of a protein play a key role in protein function.[20] Additionally, it has been shown that these fluctuations serve as a "lubricant" for larger scale motions such as perturbation of the average structure by the binding of ligands.[21], [22] This idea can further be clarified using the concept of the hierarchical arrangement of substates. Tier 0 refers to few unique conformations each corresponding to different biological functions. Each of these tier-0 substates can assume many tier-1 statistical substates, which perform the same functions at varying rates. Since motions in tier-0 occur as a result of successive transitions among substates in tier-1, Fenimore et al. suggested that freezing out these transitions would prevent the occurrence of the motions in tier-0.[23] This idea could be extrapolated to the larger scale protein motions that exceed the transitions involving protein function and extending to the conformational denaturation. Namely, suppressing the atomic motions in pico-nanosecond time range might improve the conformational stability of the protein. Indeed, this idea has been supported by studies that combine the analysis of the short-time dynamics and the measurements of enzyme activities.[24] These ideas are also supported by our findings that the dynamics of lysozyme in the nanosecond time window correlate with the experimental findings of its stability embedded in the solvents.

The dynamics of proteins are greatly influenced by hydrogen bonds.[25] More specifically, it has been shown that before the relaxation of the protein could occur, the hydrogen bonding (HB) network (between the protein-solvent) has to go through a relaxation.[26] Since the solvents under the consideration are highly capable of establishing HB network, the conformational dynamics of such protein-solvent systems will naturally be affected by the hydrogen bonds at the protein-solvent interface. In fact, it has been shown that the dynamical behavior of the HB network present between the model protein and the first solvent layer dictates the structural relaxation of the entire model protein.[14], [15] Consequently, we studied the HB network based on a set of criteria involving distances and angles among atoms forming hydrogen bonds.[27] We analyzed the hydrogen bonds using a correlation function, c(t).

$$c(t) = \frac{\langle h(t)h(0) \rangle}{\langle h \rangle}$$
 (Equation 2)

Here, h(t) is 1 when a acceptor-donor pair satisfies the hydrogen bond criteria and it is 0 otherwise. Since c(t) is time dependent, it indicates the probability that a random hydrogen bonded donor-acceptor at time zero is still bonded at time *t*. Therefore, the change of c(t) quantifies the longevity of hydrogen bonds.

Figure 2b shows c(t) for lysozyme in pure glycerol and in pure trehalose at different temperatures. The initial decline relates to the vibration of atoms and rotation of hydroxyl groups of solvent molecules which lead to short living hydrogen bonds (i.e., lifetimes < 1 ps). Therefore, they have limited influence over the dynamics of the model protein. On the other hand, the secondary decline observed at longer times relates to long-living hydrogen bonds. Since, these long-living hydrogen bonds have lifetimes that are similar to the structural relaxation of lysozyme (e.g. relaxation of S(q, t)), the secondary decline in c(t) has important consequences on the dynamics of the protein. The comparison of the hydrogen bond correlation function with S(q, t) shows a clear correlation between both functions. At low temperatures, the efficacy of the HB network between lysozyme and glycerol is more robust than the one between lysozyme and trehalose, i.e. c(t) for trehalose declines faster than for glycerol at long times. Whereas the opposite is true at 300 K. At intermediate temperatures (250 K), the strengths of HB networks for both systems are similar in line with the similar dynamics displayed by lysozyme in both solvents, Figure 2a.

These findings follow the findings of the work of Tarek and Tobias[26]. They find that the structural relaxation the protein associate with the dynamics of protein-solvent hydrogen bonds and infer that the hydrogen bonding relaxation is a priori for the structural relaxation of the protein [26].

We observe that the dynamic behavior of the protein-solvent HB network is has a determining role in the nature of the structural dynamics of the protein. We also infer that his relation can also include the bio-preserving capabilities of the solvent. To bring a deeper understanding to the effect of the HB network, we calculated the protein-solvent hydrogen bonds life-time. We calculated the occupancy and the mean HB lifetime  $(\tau_{HB})$  of the intermolecular hydrogen bonds. The occupancy means the average number of simulation steps during which a hydrogen bond existed. The averaging is done over hydrogen bonds.  $\tau_{HB}$  is the occupancy multiplied by the simulation time step and divided by the number of times that the hydrogen bond is broken. These two quantities represent the strength of the hydrogen bonds since the frequency of occurrence and the duration of a hydrogen bond indicate the level of stability of the hydrogen bond. Figure 3 shows the results of this analysis. Observe that the results for glycerol decay at a faster rate than those for trehalose with increasing temperature. These different dependencies on temperature result in stronger lysozyme-glycerol hydrogen bonds at low temperatures (T < ~250 K) and weaker hydrogen bonds at high temperatures (T >  $\sim$ 250 K). The crossover seen at around 250 K implies that both solvents are equally effective at this temperature in suppressing the dynamics of the protein. This finding supports the results shown in Figure 1, where the relative dynamics of lysozyme in different solvents merge at 250 K. A comparison of important properties in relevance to the discussion is presented below in Table 1. It is clear that the dynamical parameters (i.e.,  $\tau_{HB}$  and occupancy) of the hydrogen bond network are as expected comparing their molecular weight and  $T_g$  for these molecules at 350K. Trehalose suppress the lysozyme's dynamics more effectively. The same parameters at 150 K, on the other hand, counterintuitively switch in order and glycerol suppresses lysozyme's dynamics more effectively. This observation is similar in essence to the experimental results of the rebinding kinetics of myoglobin [10], [11]. The rebinding kinetics was found to be faster in trehalose than in glycerol at low temperatures. Therefore, it is possible that the molecular mechanism of the switching we observe in this study might also be responsible in myglobin kinetics and other biomolecules in similar systems[13].

Table 1

Molecule	MW (g/mol)	Tg (K)	τ <sub>HB</sub> at 150 K (ps)	τ <sub>HB</sub> at 350 K (ps)	Occ. at 150 K (%)	Occ. at 350 K (%)
Glycerol	92	192	780	3	95	14
Trehalose	342	388	490	8	92	52

The strong correspondence between the hydrogen bond analyses and the structural relaxation of the protein supports the idea that the HB network at the protein-solvent interface is accountable for the efficacy of glycerol at low temperatures. Lowering the temperature strengthens the intermolecular hydrogen bonds in glycerol solvent (between glycerol and lysozyme) at a faster rate than trehalose does. This is due to the fact that glycerol, a simple polyol with three hydroxyl groups attached to each carbon atom, with a T<sub>g</sub> of 192 K has significantly higher degree of mobility to be able to form intricate HB network with the protein as compared to trehalose. Trehalose (a disaccharide connected by a glycosidic linkage) with a T<sub>g</sub> of 388 K, loses its mobility at the glycosidic linkage hindering its ability to change its conformation and at the hydrogen bonding capable hydroxyl groups to be able to form and maintain long lasting hydrogen bonds to form an effective dynamics-suppressing network. Thus, even though glycerol has a significantly higher mobility in its pure form than trehalose, it is capable of suppressing protein dynamics more effectively due to its superior HB network forming capability.



**Figure 3.** Lifetime  $(\tau_{HB})$  and occupancy for hydrogen bonds formed between lysozyme and glycerol/trehalose. Occupancy is presented as percent of the total number of simulation steps.

#### 4. CONCLUDING REMARKS

We conclude that the HB network at the protein-solvent interface is responsible for the effectiveness of solvent in suppressing structural relaxation of the protein. The degree at which the solvents are able to form and maintain an intricate HB network dictates the relative effectiveness of the dynamical suppression. The effectiveness of this network is of such critical importance that it supersedes the relative internal mobility of the solvents.

It is important to note that there is a substantial difference in the time scales available to MD simulations and those involved in conformational denaturation of proteins. As the boundaries for the computational limitations expand the gap between these time scales will diminish. By then, the molecular simulations could clarify if there are other molecular mechanisms that are responsible for the conformational denaturation.

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