Molecular Packing, Hydrogen Bonding, and Fast Dynamics in Lysozyme/Trehalose/Glycerol and Trehalose/Glycerol Glasses at Low Hydration

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**Abstract**

Water and glycerol are well-known to facilitate the structural relaxation of amorphous protein matrices. However, several studies evidenced that they may also limit fast (\(\sim\) pico-nanosecond, ps-ns) and small-amplitude (\(\sim\) Å) motions of proteins, which govern their stability in freeze-dried sugar mixtures. To determine how they interact with proteins and sugars in glassy matrices and, thereby, modulate their fast dynamics, we performed molecular dynamics (MD) simulations of lysozyme/trehalose/glycerol (LTG) and trehalose/glycerol (TG) mixtures at low glycerol and water concentrations. Upon addition of glycerol and/or water, the glass transition temperature, \(T_g\), of LTG and TG mixtures decreases, the molecular packing of glasses is improved, and the mean-square displacements (MSDs) of lysozyme and trehalose either decrease or increase, depending on the time scale and on the temperature considered. A detailed analysis of the hydrogen bonds (HBs) formed between species reveals that water and glycerol may *antiplasticize* the fast dynamics of lysozyme and trehalose by increasing the total number and/or the strength of the HBs they form in glassy matrices.
Introduction

Most globular proteins are very sensitive to the various stresses that they might experience upon purification, processing and long-term storage (changes in temperature, pressure, pH, hydration level, or ionic strength). Adding solutes such as sugars, polyols, polymers, amino acids or salts is a common practice to improve their stability, both in the liquid and in the solid states. However, the molecular mechanisms by which these compounds preserve proteins from degradation have not been fully deciphered, in spite of intensive research. In the case of sugars and polyols, many studies proposed that proteins are stabilized thermodynamically by the preferential exclusion of these co-solutes from the protein/solvent interface in aqueous solution, while at low water contents proteins would be preserved both by the formation of numerous protein-solute hydrogen bonds (HBs) and by the vitrification of the mixture. Yet, there are still many open questions, in particular on how water, polyols, and sugars interact with each other and/or with proteins in complex glassy mixtures. Low-molecular-weight compounds such as water, glycerol (C\(_3\)H\(_8\)O\(_3\)), or sorbitol (C\(_6\)H\(_{14}\)O\(_6\)), are often regarded as plasticizers of carbohydrate and protein matrices, since the addition of these small molecules (sometimes referred to as diluents) usually decreases their glass transition temperature, \(T_g\), as well as their elastic moduli, and increases the free volume and the water and oxygen permeabilities. However, several studies have demonstrated that they can also act as antiplasticizers, especially at low temperatures and concentrations. Ubbink and coworkers thoroughly investigated structural and thermodynamics aspects of antiplasticization on various glassy carbohydrate matrices (maltopolymer-maltose blends, maltodextrin, etc.) and showed that the antiplasticization induced by the addition of water and/or glycerol stems from the decrease of the average hole volume, \(v_h\), as probed by positron annihilation lifetime spectroscopy (PALS), as well as from the decrease of the specific volume, \(V_{sp}\), estimated using gas pycnometry (see ref. \[26\] and references therein). Moreover, their results revealed that the antiplasticization of glassy carbohydrate and biopolymer matrices by low-molecular-weight diluents manifests as a strengthening of H-bonding interactions. By means of Fourier-transform infrared (FTIR) spectroscopy, they indeed observed a low-frequency shift of the O-H stretching vibration frequency, \(\nu_{OH}\), upon addition of low amounts of water and/or glycerol, and found a linear correlation between changes in \(\nu_{OH}\) and changes in \(v_h\), thereby evidencing the interdependence between molecular interactions and molecular packing in these H-bonding systems. Such modifications of structural properties and of H-bonding interactions probably account for the changes in the fast dynamics of glassy carbohydrate matrices described in the literature using various techniques. Cicerone and Soles evidenced by means of neutron scattering experiments that the addition of 5 wt. % of glycerol strongly reduces the mean-square displacement (MSD), \(< u^2 >\), of fast (> 200 MHz) motions of trehalose molecules (C\(_{12}\)H\(_{22}\)O\(_{11}\)) over a broad temperature range (\(~ 100-350 \text{ K}\)). They derived an effective spring constant much larger for this mixture than for pure freeze-dried trehalose and, interestingly, found an inverse relationship between the \(< u^2 >\) of glassy protein/sugar matrices and the degradation rates of the embedded enzymes (horseradish peroxidase and yeast alcohol dehydrogenase). More recently, Cicerone and Douglas confirmed such a relationship for a large series of proteins mixed with either trehalose or sucrose, and suggested that the degradation of proteins in carbohydrate glasses is governed by small amplitude, fast motions (\(\beta\)-relaxation processes) of
the glassy matrix rather than by structural, \(\alpha\) relaxation, thus showing that the antiplasticization of fast motions at sub-\(T_g\) temperatures is particularly relevant for biostabilization purposes. The pioneering results of Cicerone and Soles have triggered many studies trying to further understand the antiplasticizing effect of glycerol on trehalose, as well as the enhanced stability that such mixtures may confer to embedded proteins.

The antiplasticization of trehalose motions by glycerol has been observed at various concentrations and temperatures in experimental and numerical studies. From the secondary relaxation times, \(\tau\), obtained by means of dielectric relaxation spectroscopy measurements over broad ranges of temperature and glycerol concentration, Anopchenko et al. determined the magnitude of antiplasticization, defined as the ratio \(\tau_{TG}/\tau_T\) (\(\beta\)-relaxation time in the trehalose/glycerol, TG, mixture over that in pure trehalose, T), and found maxima in the 20-30 wt \% concentration range. Similarly, the molecular dynamics (MD) simulation results of Averett et al. showed that the \(<u^2>\) of TG mixtures is minimum between 20 and 50 wt \%. In contrast, Dirama et al. and Magazu et al. observed minima of \(<u^2>\) at a glycerol weight concentration of 5 and 2.5 \% at 300 K and 100 K, respectively. Furthermore, Weng and Elliott found by means of dynamical mechanical analysis (DMA) that the fragility index, \(m\), of dehydrated TG mixtures decreases in a non-monotonic manner in the 0-20 wt. \% range, with a local minimum between 5 and 12.5 wt. \%.

Discrepancies also emerged from studies on proteins in TG mixtures: the \(<u^2>\) of RNase was minimum at a glycerol concentration of 12.2 wt. \%. in the MD study of Curtis et al., whereas Bellavia et al. evidenced from Raman spectroscopy that the denaturation temperature, \(T_m\), of lysozyme exhibits a slight maximum at a glycerol concentration of 5 \%. The discrepancies among all these studies probably arise from the diversity of the techniques employed (neutron scattering, Raman and dielectric relaxation spectroscopies, DMA, MD simulation) to investigate TG mixtures or proteins in TG matrices, as well as from the various water contents, proteins or force fields considered in experiments and simulations. For instance, the differences that emerge among the MD studies on TG mixtures indicate a strong dependence of results on the force fields used to simulate trehalose and glycerol, which might not represent accurately enough pure trehalose and glycerol, and/or trehalose/glycerol mixtures. This is actually why Averett et al. derived specifically a force field for glycerol that reproduces satisfactorily the experimental temperature dependence of bulk glycerol’s density. Using this revised force field for glycerol, they did not observe any slight density maximum upon addition of glycerol to trehalose, in contrast to previous MD studies by Dirama et al. and by Magazu et al. at glycerol contents of 5 wt. \% and of 2.5 wt. \%, respectively. Given the discrepancies among MD studies, and because no experimental data exists on the molecular hole volume or on the specific volume (that is, on density) for trehalose/glycerol glasses, it is not possible to definitely ascribe the antiplasticizing effect of glycerol on trehalose fast motions to an improved molecular packing, as found for other carbohydrate matrices by Ubbink and coworkers. However, it probably involves to some extent changes in the HB network of trehalose. Dirama et al. actually observed that the occupancy of HBs is maximum at a glycerol content of 5 wt. \% and suggested that the minimized amplitude of trehalose motions induced by glycerol originates from the simultaneous formation of multiple T-G HBs, with glycerol molecules being sometimes able to bridge the rings of trehalose. Curtis et al. also observed that the maximum effect of glycerol correlates with a maximum number of T-G HBs. These simulation results imply
that glycerol induces a strengthening of H-bonding interactions, in line with the FTIR study of Roussenova et al. on maltooligomer matrices.\textsuperscript{23}

To further understand how glycerol and water interact with trehalose and proteins and modulate their fast dynamics in glassy mixtures, we performed MD simulations of lysozyme/trehalose/glycerol (LTG) and trehalose/glycerol (TG) matrices at various glycerol concentrations ($w_G = 0$, 5, 10, 15, and 20 wt. % of the total mass of excipients) and water contents ($h = 0.0$, 0.075, and 0.15 in g of water per g of protein or excipient). The comparison of LTG and TG matrices allows us to determine whether the improved protein stability in presence of glycerol observed experimentally\textsuperscript{28,29} simply results from the antiplasticization of trehalose motions, assuming that the protein dynamics follows that of the sugar matrix,\textsuperscript{41,42} or whether it also stems from specific protein-glycerol interactions not present in trehalose/glycerol binary mixtures. Moreover, it is necessary to consider the matrices at several low water contents, since lyophilized powders usually contain a substantial amount of residual water, which obviously modulates the concentration of glycerol at which maximal antiplasticization is observed.\textsuperscript{33} Water is indeed known to strongly modify the properties of glassy matrices, even at low concentration,\textsuperscript{15,16,19,27} and it may also play a role in the antiplasticization of carbohydrate matrices.\textsuperscript{20,21,23,24,30} In a previous comparative study of lysozyme (L), trehalose (T), and lysozyme/trehalose (LT) glassy matrices at 300 K,\textsuperscript{43} we actually evidenced that water at $h = 0.075$ and 0.15 may have an antiplasticizing effect on lysozyme and trehalose motions at the pico-nanosecond (ps-ns) time scale. In the present work, we investigate LTG and TG matrices at both 300 K and 100 K, given that antiplasticization is known to depend on temperature.\textsuperscript{33,36} We first estimate the glass transition temperature, $T_g$, of the simulated systems, next compare their molecular packing, then determine the mean-square displacements (MSDs) of lysozyme and trehalose, and finally characterize intermolecular interactions through a detailed analysis of the intermolecular HBs formed between species.
Simulation details

LTG and TG glasses were prepared from the starting structures of the lysozyme/trehalose (LT) and trehalose (T) anhydrous matrices from our previous study. Briefly, randomly selected trehalose molecules were first deleted, and glycerol and water molecules were then inserted with random positions and orientations. Each system was minimized, heated up to 700 K or 650 K, equilibrated at this temperature, then cooled down to 300 K or 100 K, and further equilibrated. Production simulations were performed at the equilibrated density in the canonical (N,V,T) ensemble for 25 ns and 5 ns at 300 K and at 100 K, respectively. All simulations were performed using the CHARMM program version c35b1. Lysozyme molecules were represented using the all-atom CHARMM22 force field with the CMAP correction for backbone dihedral angles. Trehalose and glycerol molecules were modeled with the CHARMM36 carbohydrate force field and the rigid SPC/E model was considered for water. The covalent bonds involving an hydrogen atom and the geometry of water molecules were constrained using the SHAKE algorithm. The equation of motions was integrated with a timestep of 1 fs. The Langevin piston method was employed to control temperature and pressure during simulations. van der Waals interactions were smoothly force-switched to zero between 8 and 10 Å, and Lorentz-Berthelot mixing-rules have been employed for cross-interaction terms. The particle mesh Ewald (PME) method has been used to compute electrostatic interactions. Full details on simulation parameters and on the preparation of LTG and TG glasses are provided in the Supporting Information.
Results and discussion

Glass transition temperature

Water and glycerol are well-known to decrease the glass transition temperature, $T_g$, of amorphous carbohydrate and protein matrices.\textsuperscript{15–17,19–25,56} As a means to estimate the $T_g$ of the simulated LTG and TG systems, we determined the temperature dependence of their density, $\rho$, upon heating at a rate of 0.05 K/ps (see Figure 1a and Figure S1 in the Supporting Information), which we then fitted in a similar way as in Averett \textit{et al.}\textsuperscript{37} (see details and examples of fits in Figure S2 in the Supporting Information). The $T_g$ of LTG and TG systems determined in this way decreases significantly when the amount of water and/or glycerol increases (Figure 1b), in fair agreement with simulation\textsuperscript{32,34,37} and experimental\textsuperscript{25,28,56} data from literature (Figure 1c). For example, the $T_g$ of anhydrous TG mixtures decrease from 451 ± 5 K to 390 ± 3 K in the present simulations and from 388.8 ± 0.6 K to 310.8 ± 1.0 K in the DMA study of Weng and Elliott\textsuperscript{25} when $w_G$ increases from 0 to 20 % (Figure 1c). To our knowledge, no such data exist for LTG sytems. However, Padilla and Pikal determined the $T_g$ of the 1:1 L/T freeze dried mixture (LTG matrix with $w_G = 0 \%$) to be 404.9 K from Modulated DSC measurements.\textsuperscript{57} Furthermore, we can estimate the $T_g$ of 1:1 L/T mixtures to decrease from 363 K to 287 K when $h$ raises from 0.0 to 0.15, using the Gordon-Taylor parameters obtained by Bellavia \textit{et al.}\textsuperscript{22} By comparison, the $T_g$ of the corresponding simulated mixture decreases from 598 ± 6 K to 481 ± 3 K (Figure 1b). Clearly, the $T_g$ of simulated LTG and TG mixtures exceed those determined experimentally.\textsuperscript{22,25,28,57} Such differences arise in part from the extremely high heating or cooling rates used in MD simulations (0.05 K/ps in this study, that is, 3.10$^{12}$ K/min) in comparison with those used experimentally (typically ~ 10 K/min).\textsuperscript{32,34,58} They also stem from the various simulation protocols and force fields employed in MD simulations, which, for instance, lead to different densities (see next section). Besides, at a given content, the presence of water induces a larger decrease of the $T_g$ of LTG and TG systems than that of glycerol (Figure 1b), in line with experimental data,\textsuperscript{25,28,56} thereby suggesting that water interacts more strongly with lysozyme and trehalose than glycerol does. It must also be pointed out that the density of anhydrous LTG mixtures is slightly larger for $w_G = 5 \%$ than for $w_G = 0 \%$ at temperatures below ~ 400 K (Figure 1a). To understand the origin of this behavior, we have investigated the influence of water and glycerol on the density and the free volume of LTG and TG systems at 300 K, that is, in their glassy state.
Figure 1: (a) Temperature dependence of the density, $\rho$, of anhydrous LTG mixtures at $w_G = 0$, 5, and 20 % upon heating from 50 to 800 K at a rate of 0.05 K/ps. The inset shows a zoom on the [75-125 K] temperature range to evidence the higher $\rho$ of the anhydrous LTG matrix with $w_G = 5$ % at temperatures below $\sim$ 400 K. (b) Glass transition temperature, $T_g$, of LTG and TG systems as a function of the glycerol content, $w_G$, and for the different hydration levels $h$. (c) Comparison of the $T_g$ for the anhydrous TG mixtures obtained in this work with those determined in previous MD simulation$^{32,34,37}$ and experimental$^{25,28}$ studies.
Molecular packing

Figure 2a-b shows the densities, \( \rho \), of the LTG and TG matrices at 300 K as a function of the glycerol concentration, \( w_G \), at various hydration levels, \( h \). The dependence of \( \rho \) on \( w_G \) and \( h \) is strikingly different whether lysozyme is present or not: whereas \( \rho \) decreases monotonically when \( w_G \) or \( h \) increases in the TG mixtures (Figure 2b), small maxima systematically emerge for the LTG glasses at glycerol contents of 5-10 wt. % (Figure 2a), whatever the water content, \( h \), considered (the largest density increase occurs in the anhydrous LTG mixture upon the addition of 5 % of glycerol and is about 0.005 g.cm\(^{-3}\)). The decrease of density of TG mixtures with \( w_G \) is expected when considering the smaller density of liquid glycerol (\( \sim 1.26 \) g.cm\(^{-3}\)) compared to that of amorphous trehalose (\( \sim 1.50 \) g.cm\(^{-3}\)). It is consistent with that found by Averett et al. with the original OPLS force field for trehalose and an optimized version for glycerol, even though the densities of our mixtures are downshifted by about 0.1 g.cm\(^{-3}\) (Figure 2c). This difference probably stems from the CHARMM36 carbohydrate force field, which underestimates the densities of concentrated solutions and crystals of carbohydrates as well as the density of bulk glycerol. In contrast, slight density maxima appear at \( w_G \) of 5 and 2.5 % in the MD studies of Dirama et al. and of Magazu et al., respectively (Figure 2c), as well as at \( w_G = 5 \) % in that of Riggleman and de Pablo, for temperatures below ~ 250-280 K (see Figure 1 in ref. [34]). The presence of a maximum in the dependence of the density of TG mixtures on \( w_G \) thus relies on the force fields used in MD simulations to represent trehalose and glycerol. Experimental density data would help to capture such a subtle effect and to improve force fields accordingly. Nonetheless, the distinct density behaviors of LTG and TG matrices in the present study reveal substantial differences in the relative packing efficiencies of lysozyme and trehalose molecules: excluded volume effects are much stronger in the presence of lysozyme than in its absence, owing to the globular shape and the much larger size of lysozyme in comparison with trehalose (radii of gyration of about 14.3 and 3.5 Å, respectively), which obviously reduce its packing efficiency. As a consequence, the density of amorphous trehalose exceeds that of freeze-dried powders of proteins of various molecular weights - human growth hormone (22 kDa), bovine serum albumin (66 kDa), or immuno globulin G (150 kDa) - determined using gas pycnometry. This rationalizes why the densities of TG mixtures are systematically larger than those of LTG ones, at given glycerol and water contents. Besides, the increases in the density of LTG mixtures with \( w_G \) and \( h \) may be ascribed to the small sizes of water and glycerol, which can thereby access volumes inaccessible to the bigger trehalose (note that the mean molecular volumes occupied by water, glycerol, and trehalose are about 30, 120, and 380 Å\(^3\) in the bulk, assuming that their bulk densities are 1.0, 1.26, and 1.50 g.cm\(^{-3}\) respectively). Interestingly, the increase of \( \rho \) when \( h \) increases from 0.0 to 0.075 is significantly larger than that found when \( w_G \) raises from 0 to 5-10 %, suggesting that, owing to its smaller size, water may enter regions at the protein-matrix interface that are sterically inaccessible to glycerol.

The non-monotonic dependence of \( \rho \) on \( w_G \) and \( h \) in the LTG mixtures clearly suggests that glycerol and water can act as antiplasticizers at low concentrations. MD simulations of a coarse-grained polymer indeed indicated that antiplasticization is linked to enhanced packing in the glassy state. To further study the influence of glycerol and water on the molecular
packing of LTG and TG matrices, we then determined their free volume fraction, $f$, in a similar way as in ref. [64] (Figure 2d-e). $f$ steadily decreases upon addition of glycerol, in line with the positron annihilation lifetime spectroscopy (PALS) and Fourier transform infrared (FTIR) study of Roussenova et al. on maltodextrin-glycerol amorphous matrices, which revealed that the addition of glycerol at concentrations up to 20 wt. % reduces nonlinearly the average molecular hole size, $v_h$, thereby enhancing molecular packing.[23] $f$ also diminishes with $h$ in the LTG and TG matrices, which seems consistent with the decrease of hole volume found for glassy maltopolymer-maltose and maltodextrin-glycerol matrices upon sorption of low contents of water (up to weight fractions of $\sim 0.04-0.08$). [20,21,23,26] The larger $f$ values for LTG matrices in comparison with TG ones may be ascribed to a greater structural disorder when mixing molecules of significantly different sizes and also, to a smaller extent, to internal protein cavities. This difference in molecular packing between LTG and TG mixtures probably explains why $f$ keeps decreasing when $h$ increases from 0.075 to 0.15 for a given glycerol content in the LTG matrices, while $f$ hardly changes with $h$ in the TG matrices for the same increase of water content.
Figure 2: Left panel: Density, $\rho$, of the LTG (a) and TG (b) glasses as a function of the glycerol content, $w_G$, and the hydration level, $h$, at 300 K. The densities of the anhydrous TG glasses obtained in this work are compared with those determined in previous MD simulation studies in (c). Right panel: Free volume fraction, $f$, of the LTG (d) and TG (e) glasses as a function of the glycerol content, $w_G$, and the hydration level, $h$, at 300 K. $f$ denotes the ratio of the total free volume accessible to a probe radius of 0.5 Å, $V_f$, to the total volume of the simulation box, $V$ ($f = 100*V_f/V$). Rather similar results for $\rho$ and $f$ were obtained at 100 K (see Figure S3 in the Supporting Information).
In order to get a more comprehensive insight into the molecular packing of LTG and TG mixtures, we also computed the distribution of hole volumes in these systems (Figure 3). The addition of glycerol globally tends to reduce the number of holes larger than $\sim 30 \, \text{Å}^3$. This effect clearly appears in the TG matrices, in which the absence of lysozyme may facilitate the packing between trehalose and glycerol molecules. In contrast, the number of holes bigger than $\sim 200 \, \text{Å}^3$ is slightly larger at $w_G = 5 \%$ than at $w_G = 0 \%$ for the anhydrous LTG matrix, which may indicate a lack of equilibration of LTG matrices. Moreover, in agreement with the results shown in Figure 2d-e, the shift of distributions towards smaller hole volumes is more pronounced upon addition of water than when adding glycerol. This result can obviously be ascribed to the smaller size of water, which can intercalate more easily between lysozyme, trehalose, and glycerol molecules. Note, however, that the shift to low hole volumes cannot be simply explained by a "hole filling" mechanism, as discussed by Roussenova et al. in ref. [21] (see also a short discussion and Figure S5 in the Supporting Information).
Figure 3: Probability distribution functions of the size of free volumes accessible to a probe radius of 1.0 Å, \( P(V) \), at 300 K: (a) in the anhydrous LTG matrices at \( w_G = 0, 5, \) and 20 \( \% \) and, for comparison, in the anhydrous TG matrix at \( w_G = 0 \% \), (b) in the LTG matrices at \( w_G = 5 \% \) and hydration levels, \( h \), of 0.0, 0.075, and 0.15. The corresponding distributions for the TG matrices are shown in insets. Pictures on the spatial distributions of free volume in representative configurations of LTG and TG matrices at 300 K are provided in Figure S4 in the Supporting Information.

As a way to describe how glycerol and water modify the local free volume of lysozyme and trehalose in the LTG and TG glasses, we then determined their average molecular volumes, \( \bar{V} \), using Voronoi tessellation\(^66\) (Figure 4). \( \bar{V} \) of lysozyme and trehalose tend to decrease when \( w_G \) and/or \( h \) increase in the simulated systems. The decreases are particularly steep in the anhydrous LTG matrix when \( w_G \) increases from 0 to 5 wt. \% (\( \sim -1 \% \)) or when \( h \) increases from 0.0 to 0.075 (\( \sim -2 \% \)). These results definitely indicate that glycerol and water enhance the packing of lysozyme and trehalose molecules, consistent with Figures 2 and 3. Nevertheless, the molecular volumes of lysozyme and trehalose in LTG and TG matrices exceed those found experimentally in dilute aqueous solutions at room temperature\(^67,68\) by \( \sim 7-11 \% \) for lysozyme in LTG systems and by \( \sim 14-21 \% \) and \( \sim 11-16 \% \) for trehalose in LTG and TG matrices, respectively. The partial molar volumes of lysozyme and trehalose were indeed determined to be 10280 cm\(^3\).mol\(^{-1}\) \( \approx 17070 \text{ Å}^3 \) for lysozyme\(^67\) and \( \sim 210 \text{ cm}^3 \text{.mol}^{-1} \approx 350 \text{ Å}^3 \) for trehalose.\(^68\) These differences probably stem in part from the underestimated densities of simulated systems. But, they could also indicate that both lysozyme and trehalose are not as well solvated in the studied glasses as they are in dilute aqueous solutions, owing to significant excluded volume effects that do not allow lysozyme to interact as intimately with trehalose and glycerol as it interacts with water. Similarly, the larger \( \bar{V} \) of trehalose in the LTG matrices than in the TG ones at given water and glycerol contents shows that trehalose cannot interact as efficiently with lysozyme as it interacts with itself.
Figure 4: Average molecular volumes, $\bar{V}$, of lysozyme (a) and trehalose (b) in the LTG glasses at 300 K, as a function of the glycerol content, $w_G$, and for the different hydration levels, $h$. $\bar{V}$ of trehalose in the TG glasses at 300 K are displayed in (c). $\bar{V}$ were computed using Voronoi tessellation as performed by the program of Gerstein et al.\textsuperscript{66} For clarity, the standard deviations around mean values are not shown as error bars, but are given in Table S3 in the Supporting Information.
Mean-square displacements

The improved molecular packing of LTG and TG glasses induced by the addition of glycerol and/or water (Figures 2-4) suggests that glycerol and water also decrease the short-time scale MSD of LTG and TG mixtures, since a relationship between the free volume and the Debye-Waller (DW) factor was found for glass-forming systems (see, for instance, ref. [69, 70]). Figure 5a shows the time dependence of the MSD of the hydrogens of lysozyme in the anhydrous LTG matrices with $w_G = 0$ and 10%. The plateau that appears at a time of 1-2 ps reflects vibrational and rattling motions of atoms within the cage formed by their neighbors, while the steep increase that follows arises from anharmonic motions. Hong et al. identified three classes of motions for the nonexchangeable hydrogen atoms of lysozyme: "localized diffusion", "methyl group rotations", and "jumps". The distinct time dependences of the MSDs of lysozyme’s methyl, hydroxyl, and H$_{\alpha}$ backbone hydrogen atoms illustrate such a great heterogeneity of motions (inset of Figure 5a). The detailed analysis of such motions is beyond the scope of this study, so that we will only consider and discuss the influence of water and glycerol on the average MSDs of lysozyme’s and trehalose’s hydrogen atoms in the following. Figure 5a evidences that the presence of glycerol can decrease the MSD of lysozyme in the ps-ns time scale. In contrast, water is found to slightly decrease the MSD of lysozyme in the plateau regime ($\sim 1$ ps), whereas it increases it at longer time scales ($\sim 100$ ps for the LTG matrix with $w_G = 10\%$ and $h = 0.15$, Figure 5b). Figure 5c-d shows the corresponding MSDs at 100 K, at which the thermal activation of methyl group rotations and of jumps is inhibited. At such a low temperature, both glycerol (Figure 5c) and water (Figure 5d) reduce the MSD of lysozyme from the ps to the ns time scale.

In order to investigate in a systematic and straightforward manner the effects of water and glycerol on the MSDs of lysozyme and trehalose in LTG and TG matrices, we then compared MSDs at two time scales, 1 ps and 1 ns, both at 300 K and at 100 K (Figure 6). In LTG matrices, the addition of glycerol tends to lower the MSDs of L and T at both time scales and temperatures (with a few exceptions, where the MSDs increase moderately, usually within few standard deviations), thereby indicating that glycerol may act as an antiplasticizer on lysozyme and trehalose fast motions in this concentration range. This is in fair agreement with the neutron scattering study of Tsai et al., who found that the addition of glycerol lowers the amplitude of motions of Lysozyme/Glycerol mixtures (80 wt. %/20 wt. %) with respect to those of dry lysozyme at temperatures below the so-called dynamic transition temperature, $T_d$, of 330 K for this mixture. Besides, antiplasticization of trehalose motions by glycerol is absent in TG matrices at $h = 0.15$ (Figure 6i-j). It emerges only at lower water contents, rather modestly at 300 K (Figure 6i-j), but much more clearly at 100 K (Figure 6k-l). This result appears at variance with the neutron scattering data of Cicerone and Soles, which evidenced a significant decrease of the MSD of trehalose in TG mixtures at $w_G = 5\%$ over a broad temperature range ($\sim 100$-350 K). But, the modest decrease of MSD induced by glycerol in TG matrices fairly agrees with later studies by Riggleman and de Pablo, Magazu et al., and by Averett et al. Furthermore, the strong temperature dependence of the antiplasticizing effect of glycerol on trehalose motions appears consistent with that experimentally found by Anopchenko et al. in TG matrices from dielectric relaxation spectroscopy. Besides, the influence of water on the fast motions of lysozyme and trehalose in LTG and TG matrices is also complex and strongly depends on the time scale.
Figure 5: Time dependence of the mean-square displacement (MSD) of the hydrogens of lysozyme in LTG matrices: (a) at \( h = 0.0 \) and \( w_G = 0 \) or 10\%, (b) at \( w_G = 10 \% \) and \( h = 0.0 \) or 0.15. The corresponding MSDs at 100 K are shown in (c) and (d), respectively. The inset of (a) shows the MSDs of three kinds of hydrogens of lysozyme in the anhydrous LTG matrix at \( w_G = 0 \% \): (i) methyl groups, \( H_{CH_3} \) (from ALA, ILE, LEU, MET, THR, and VAL residues), (ii) hydroxyl groups, \( H_{OH} \) (from SER, THR, and TYR residues), and (iii) \( H_\alpha \) backbone atoms of lysozyme. The inset of (b) shows a zoom on the 0.5-3 ps time range to make clearer the decrease of the MSD of lysozyme induced by the addition of water. The time dependences of the MSDs of lysozyme, trehalose, glycerol, and water in LTG and TG glasses at \( w_G = 10 \% \) and \( h = 0.15 \) are provided in Figure S6 in the Supporting Information.
and temperature considered: at 300 K, water diminishes the MSDs of L and T at the ps time scale (only at $h=0.075$ in TG matrices, Figure 6a,e,i), but raises them at the ns time scale (Figure 6b,f,j). In contrast, water globally tends to decrease the MSDs of both L and T at the ns time scale at 100 K (Figure 6d,h,l), thereby demonstrating that it stiffens vibrational, (quasi-)harmonic motions at low temperatures. These results corroborate those of Nickels et al. from neutron scattering measurements on green fluorescent protein (GFP) which showed that the MSD of GFP at a hydration level, $h$, of 0.4 g(D$_2$O)/g(protein) is either enhanced or reduced with respect to that of the dry protein for temperatures larger or lower than $\sim 240$ K, respectively. They are also consistent with those of Hong et al., who showed that the effective force constant derived from MD simulation as well as the frequency of longitudinal sound waves, $\nu_L$, determined from Brillouin light scattering experiments are larger for hydrated than for dry GFP at temperatures below $\sim 180$ K. Analogous temperature-dependent effects of water on the MSD of RNase A were also found by Tarek and Tobias from MD simulation.

Finally, we checked whether the changes of MSD of LTG and TG matrices upon the addition of glycerol or water are related to changes in density. For this purpose, we assumed that the MSD at a given glycerol or water content, $x$ ($x = w_G$ or $h$), may be written as $MSD(x) = MSD(0) + \alpha.(\rho(x) - \rho(0))$, where $MSD(0)$ and $\rho(0)$ correspond to the MSD and density in the absence of the considered diluent, and $\alpha$ is a constant (see further details in the Supporting Information). Although very simple, this phenomenological equation is able to qualitatively account for the minima in the MSDs of LTG matrices or for the increase of the MSD of TG ones observed at the ps time scale upon addition of glycerol at 300 K (see Figure S7a,e in the Supporting Information). Moreover, it clearly relates the antiplasticizing effect of water on the ps-time scale motions of LTG matrices at 100 K to the corresponding increase of density (see Figure S8c in the Supporting Information). Nevertheless, a much deeper analysis would be required to explain on theoretical grounds how MSD depends on density in such systems.

In the following, we will describe thoroughly the intermolecular hydrogen bonds formed between species to clarify the complex dependences of the MSDs of lysozyme and trehalose on the concentrations of glycerol and water, on temperature, and on the time scale considered.
Figure 6: Mean-square displacements (MSDs) of the hydrogens of lysozyme and trehalose in LTG and TG glasses as a function of the glycerol content, wG, and for the different hydration levels, h: (a-d) L in LTG, (e-h) T in LTG, and (i-l) T in TG matrices. MSDs were determined at two time scales, 1 ps and 1 ns, for the two temperatures considered, 300 K (left two panels) and 100 K (right two panels). Error bars correspond to standard deviations from mean values determined by splitting trajectories into 5 sub-trajectories for all MSDs at 300 K and for MSDs at 100 K at the ps time scale, while 3 sub-trajectories were considered for MSDs at 100 K at the ns time scale.
Hydrogen bonds

Previous studies evidenced that the stability of proteins in the solid state is improved when they form numerous HBs with excipients (see e.g. ref. [12] and reviews in ref. [2, 4, 5]). It has been proposed that these HBs substitute for those that proteins form in aqueous solution with their hydration water and, thereby, maintain the native structure and function of proteins in the dry state (water replacement hypothesis). Furthermore, the antiplasticizing effect of glycerol on trehalose has been correlated with the formation of T-G HBs in previous MD studies,[32,38] even though antiplasticization has also been observed in model systems that do not form HBs.[78] Thus, we characterized thoroughly the distribution of HBs between the different species, as well as their geometry and dynamics, to deeply understand the complex influences of glycerol and water on the fast motions of lysozyme and trehalose observed in Figures 5-6. For this purpose, we determined the intermolecular HBs formed between the different species following the same geometric criterion that we used previously: a hydrogen bond between a pair of donor, D, and acceptor, A, atoms was considered to exist if the D···A distance was less than 3.4 Å and if the D-H···A angle was larger than 120°. Figure 7a-c shows the total numbers of intermolecular HBs, \( n_{\text{HB}} \), that lysozyme, trehalose, and glycerol form in the LTG mixtures, as a function of the glycerol content, \( w_G \), and the hydration level, \( h \). The \( n_{\text{HB}} \) of both lysozyme and trehalose increase much more with \( h \) than with \( w_G \). This difference obviously stems from the smaller size and simpler topology of water compared to glycerol, which allow a more intimate interaction with lysozyme and trehalose. It is fully consistent with the larger decreases of the molecular volumes \( V \) of L and T found when adding water than upon addition of glycerol (Figure 4). Moreover, the \( n_{\text{HB}} \) of glycerol increases systematically with \( h \) and tends to increase with \( w_G \) at \( h = 0.0 \) and 0.075. Besides, Figure 7a demonstrates that lysozyme remains only partially hydrated in the LTG matrices, as we previously found for LT matrices in ref. [43]. This indicates that trehalose and glycerol are unable to fully replace the HBs that lysozyme forms in dilute aqueous solutions (we found that lysozyme forms about 330 HBs with water in ref. [79]). Therefore, the water replacement hypothesis is only valid to some extent. Furthermore, at given \( w_G \) and \( h \), trehalose forms about two HBs less in LTG matrices than in TG ones (see Figure S9 in the Supporting Information). This can be ascribed to the disorganization of the molecular packing of trehalose molecules induced by lysozyme and it corroborates the larger molecular volumes \( V \) of trehalose in LTG matrices (Figure 4b-c). The roughness of the protein surface as well as excluded volume effects between neighboring proteins reduce efficient protein-sugar H-bonding interactions. This result appears compatible with the lower density and larger free volume fraction of LTG systems compared to TG ones (Figure 2). The lower \( n_{\text{HB}}(T) \) in LTG matrices is also consistent (i) with the results of Carpenter and Crowe, which showed that the capacity of carbohydrates for hydrogen bonding decreases when they are mixed with lysozyme,[10] and (ii) with those of Katayama et al., who found a positive excess enthalpy when mixing RNase A with trehalose or sucrose, thereby suggesting weaker or fewer HBs in the protein/sugar mixtures than in the two pure components.[80]

The decomposition of \( n_{\text{HB}} \) into contributions from each species provides useful information (Figure 7d-l and Table S4 in the Supporting Information). First, the numbers of L-T and L-W HBs clearly overwhelm that of L-L HBs. This may explain why protein aggregation during storage is reduced in presence of trehalose, which physically separate proteins from...
Moreover, Figure 7d-f reveals that the increase of \( n_{HB}(L) \) with \( w_G \) and \( h \) stems from the substitution of L-T HBs by L-G and/or L-W ones. Similarly, the \( n_{HB} \) of trehalose in LTG and TG matrices increase much more with \( h \) than with \( w_G \) (see Figure S9 in the Supporting Information), which obviously stems from the more significant substitution of T-T HBs by T-W HBs than by T-G ones (see Tables S4 and S5 in the Supporting Information). In addition, Figure 7d-f and Table S4 prove that trehalose, glycerol, and water are not homogeneously distributed around lysozyme in LTG matrices. Indeed, L-W HBs represent about 45-49 % (depending on \( w_G \)) of \( n_{HB}(L) \) at \( h = 0.15 \), even though water amounts for only \( \sim 15 \) % of the total mass of the solvent in these systems. Therefore, lysozyme interacts preferentially with water rather than with trehalose. Furthermore, the proportion of HBs involving lysozyme (L-L, T-L, G-L, and W-L) increases when the size of species decreases, thereby evidencing excluded volume effects: L-L interactions are much less likely than W-L ones, since water may interact intimately with lysozyme. Accordingly, trehalose and glycerol form much more HBs with other trehalose and/or glycerol molecules than with lysozyme.

Figure 7: Total numbers of intermolecular HBs, \( n_{HB} \), formed by lysozyme, \( n_{HB}(L) \) (a), trehalose, \( n_{HB}(T) \) (b), and glycerol, \( n_{HB}(G) \) (c) in the LTG matrices as a function of the glycerol content, \( w_G \), for the different hydration levels, \( h \), at 300 K. The decompositions of \( n_{HB}(L) \), \( n_{HB}(T) \), and \( n_{HB}(G) \) into contributions from HBs involving each species are shown in d-f, g-i, and j-l, respectively (the corresponding numerical values are provided in Table S4 in the Supporting Information). Moreover, the \( n_{HB} \) of lysozyme and trehalose in LTG matrices at 100 K, as well as the \( n_{HB} \) of trehalose in TG matrices at 300 K and at 100 K are shown in Figure S9 in the Supporting Information.
Preferential interactions

Given that Figure 7d-l suggests that trehalose, glycerol, and water have different affinities for lysozyme, we characterized these differences quantitatively by determining the fractions of HBs formed by trehalose, glycerol, and water with lysozyme, 
\[ f_{HB}(L-X) = \frac{n_{HB}(L-X)}{n_{HB}(L-T) + n_{HB}(L-G) + n_{HB}(L-W)}, \]
where X stands for the considered species (X = T, G, or W). We then normalized these fractions by the fractions of HBs, 
\[ f_{HB,theor.}(L-X) = \frac{n_{HB,theor.}(L-X)}{n_{HB,theor.}(L-T) + n_{HB,theor.}(L-G) + n_{HB,theor.}(L-W)}, \]
that species X (trehalose, glycerol, or water) could form with lysozyme assuming that each hydroxyl group of trehalose and glycerol may form three HBs with lysozyme (one as donor and two as acceptors) and each water molecule four (two as donors and two as acceptors). Under this hypothesis, 
\[ n_{HB,theor.}(L-T) = 3*8*N_T, \]
\[ n_{HB,theor.}(L-G) = 3*3*N_G, \]
\[ n_{HB,theor.}(L-W) = 4*N_W, \]
where \( N_T, N_G, \) and \( N_W \) denote the numbers of trehalose, glycerol, and water molecules, respectively, in the simulation box (see Table S1 in the Supporting Information). The normalized ratio 
\[ r_{HB}(L-X) = \frac{f_{HB}(L-X)}{f_{HB,theor.}(L-X)} \]
then indicates whether species X forms more HBs than the one expected \((r_{HB}(L-X) > 1)\) or not \((r_{HB}(L-X) < 1)\). Figure 8 displays the ratios \( r_{HB}(L-G) \) and \( r_{HB}(L-W) \) for the different simulated LTG systems. In the absence of water, glycerol interacts preferentially with lysozyme in comparison with trehalose (an excess from about 40 % for \( w_G = 5 \% \) down to \( \sim 20-25 \% \) for \( w_G \geq 15 \% \) is observed for L-G HBs). This definitely shows that glycerol is not homogeneously distributed in the LTG matrices, but rather that it is found slightly preferentially in the vicinity of the protein surface. This result can be ascribed to the smaller size of glycerol, which can therefore fit in empty spaces at the protein-matrix interface from which the bigger trehalose is size-excluded, and it explains the decrease of \( V \) of lysozyme when \( w_G \) increases (Figure 4a). Upon addition of water \((h = 0.075-0.15)\), \( r_{HB}(L-G) \) decreases significantly, so that glycerol forms within 10-15 % of the number of HBs with lysozyme that one would expect. Conversely, water forms a great excess of HBs with lysozyme, consistent with the preferential hydration hypothesis \( ^{35} \) in dilute or semi-dilute solutions and with the water entrapment hypothesis \( ^{83} \) in the solid state. This result corroborates those from previous MD studies on lysozyme and myoglobin in aqueous carbohydrate solutions and concentrated matrices. \( ^{45,79,84–86} \) In addition, \( r_{HB}(L-W) \) increases when \( h \) decreases, in agreement with previous simulation results where the preferential hydration of lysozyme in aqueous disaccharide solutions was found to increase with the sugar concentration. \( ^{79} \) This supports the water anchorage hypothesis, \( ^{57,88} \) which suggests that the role of residual water is to anchor the dynamics of proteins to that of the embedding matrix. It is worth mentioning that the preferential exclusion of trehalose from the surface of lysozyme with respect to glycerol probably originates solely from larger topological constraints and sterical hindrance effects on trehalose, rather than from differences in the affinity of their respective hydroxyl groups for lysozyme. Indeed, the hydroxyl groups of trehalose and glycerol share the same non-bonded parameters for electrostatic and van der Waals interactions in the CHARMM36 force field used in the current study. \( ^{47–49} \) In contrast, the preferential hydration of lysozyme probably does not exclusively results from topological and excluded volume effects, but could also arise from a greater chemical affinity of water for lysozyme, since, for instance, water exhibits a larger dipole moment than that of the hydroxyls of glycerol and trehalose molecules (the hydroxyl oxygen and hydrogen atoms of both trehalose and glycerol carry partial charges of -0.65 e and +0.41 e, respectively, while the partial charges on water...
oxygen and hydrogen atoms are $-0.8476 \, e$ and $+0.4238 \, e$, respectively). Furthermore, it is important to keep in mind that the preferential hydration of lysozyme described above does not imply that trehalose remains far from the surface of lysozyme. About 80 to 90 % of trehalose molecules actually form at least one HB with lysozyme in the LTG systems, depending on the water and glycerol contents considered (data not shown), and may thus be considered as interfacial. Therefore, distribution inhomogeneities of trehalose and glycerol around lysozyme are short-range, and preferential hydration in such concentrated protein matrices reflects their inability to form HBs with lysozyme as efficiently as water does rather than any phase separation.

Figure 8: Ratio $r_{HB}(L-X)$ for L-G and L-W HBs as a function of the glycerol content, $w_G$, for different hydration levels, $h$, at 300 K. $r_{HB}(L-X)=f_{HB}(L-X)/f_{HB,\text{theor.}}(L-X)$, where $f_{HB}(L-X)=n_{HB}(L-X)/(n_{HB}(L-T)+n_{HB}(L-G)+n_{HB}(L-W))$, and $X$ stands for the considered species ($X = T, G, \text{ or } W$). $f_{HB,\text{theor.}}(L-X)$ denotes the fraction of HBs that species $X$ (trehalose, glycerol, or water) could form with lysozyme assuming that each hydroxyl group of trehalose and glycerol may form three HBs with lysozyme (one as donor and two as acceptors) and each water molecule four (two as donors and two as acceptors). By definition, $r_{HB}(L-X)$ indicates whether species $X$ forms an excess ($r_{HB}(L-X)>1$) or a lack ($r_{HB}(L-X)<1$) of HBs with lysozyme.
Besides, the preferential interaction of lysozyme with water and, to a lower extent, with glycerol rather than with trehalose implies that glycerol and water can form bridges between lysozyme and trehalose molecules (see Figures S10 and S11 in the Supporting Information). Interestingly, close to 80% of glycerol molecules are shared between lysozyme and trehalose in the anhydrous LTG mixture at \( w_G = 5 \% \), which may explain why the MSD of lysozyme at 300 K is minimal in this system (Figure 6a-b), since glycerol locates primarily at the protein-sugar interface and is therefore likely to improve the protein-matrix coupling. Finally, we analyzed glycerol molecules that bridge the two rings of trehalose (see Figures S12 and S13 in the Supporting Information), following the hypothesis made by Dirama et al.\(^{32}\) that such bridges would constrain trehalose motions. However, we did not find any straightforward relationship between such HB patterns and the effect of glycerol on the fast dynamics of trehalose.

**Geometry of HBs**

The chemical heterogeneity of the polar groups involved in HBs (hydroxyls, carbonyls, carboxylates, etc.) implies that the HBs formed by lysozyme, trehalose, glycerol, and water are characterized by various geometries, strengths, and dynamics that are not accounted for by the analysis performed above. In Figure 9, we present the distance and angle distributions of various HBs formed in the LTG matrices. Interestingly, L-G HBs are, on average, shorter and more linear than L-T ones in the anhydrous LTG matrix at \( w_G = 5 \% \) (Figure 9a-b). Given that the hydroxyl hydrogen and oxygen atoms of glycerol and trehalose share the very same non-bonded parameters in the CHARMM36 force field used in the present study,\(^{47–49}\) these differences in the geometry of HBs reflect differences in sterical and/or topological constraints. This supports that glycerol interacts more intimately with lysozyme than trehalose does, owing to its smaller size and less complex topology. This result probably explains why the addition of glycerol reduces the MSD of lysozyme, particularly at the ps time scale (see Figure 6a-b). Figure 9a-d reveals that the distance and angle distributions of L-G HBs in LTG matrices at \( h = 0.0 \) hardly change with the glycerol content. Nonetheless, it is interesting to notice that the L-G HBs are very slightly shorter and/or more linear, and therefore slightly stronger, for \( w_G = 5 \% \). This may actually rationalize the minimum of MSD(L) at \( w_G = 5 \% \) found in Figure 6a. Finally, Figure 9e-f evidences that L-W HBs are significantly shorter and more linear, and thus much stronger, than L-T HBs (in line with our previous study\(^{43}\)) and than L-G ones. This result confirms the suggestion of Roussenova et al.\(^{23}\) that water molecules may form stronger HBs than the OH groups of carbohydrates, owing to the fewer constraints they experience for their intermolecular interactions. It likely explains the antiplasticizing effect of water observed on the fast motions of lysozyme (see Figure 6) and trehalose (we also found that T-W HBs are stronger than T-T ones, see for instance the results obtained for TG matrices in Figure S15 in the Supporting Information), since the addition of water increases both the number (see Figure 7 and Table S4 in the Supporting Information) and the strength of the intermolecular HBs formed by lysozyme and trehalose in the LTG matrices. It must also be pointed out that the stronger L-W HBs compared to L-T and L-G ones probably accounts in part for the preferential interaction of lysozyme with water, beyond the straightforward larger sterical and topological constraints experienced by trehalose and glycerol compared to water.
Figure 9: Distributions of the donor-acceptor distance (left panel) and donor-hydrogen-acceptor angle (right panel) of various HBs formed in the LTG matrices at 300 K: (a-b) L-T and L-G HBs at $h = 0.0$ and $w_G = 5\%$, (c-d) L-G HBs at $h = 0.0$ and $w_G = 5, 10, 15, \text{and} 20\%$, (e-f) L-T, L-G, and L-W HBs at $h = 0.15$ and $w_G = 20\%$. Rather similar distributions were observed at 100 K (see Figure S14 in the Supporting Information).
Dynamics of HBs

The various geometries of the HBs formed in LTG and TG matrices imply a high diversity of dynamics, which needs to be considered to understand the plasticizing and antiplasticizing effects of glycerol and water on the fast motions of lysozyme and trehalose. In this study, we investigated the dynamics of HBs using the time autocorrelation function, $C_{HB}(t)$, defined as $C_{HB}(t) = \langle b(0).b(t) \rangle / < b >$, where $b(t)$ is 1 if a D-H···A HB between a given set of donor, D, hydrogen, H, and acceptor, A, atoms exists at time $t$, and is zero otherwise. The brackets mean averaging over the different pairs of HBs and time origins. By definition, $C_{HB}(t)$ relates to the probability that a HB formed at time 0 still exists at time $t$, even if it has broken in between. The short-time decay of $C_{HB}(t)$ originates from fast motions such as librations or intermolecular vibrations, and therefore denotes the strength of the HBs considered. In contrast, the long-time behavior of $C_{HB}(t)$ results from the relative diffusion of the donor and acceptor atoms involved in the HBs and, thus, describes the structural relaxation of HBs. The slower decay of $C_{HB}$ for L-G HBs than for L-T ones at time scales shorter than about 10 ps in the anhydrous LTG matrix at $w_G = 5$ % confirms that L-G HBs are stronger than L-T ones in this system (Figure 10a), consistent with the results from Figure 9a-b. This explains why the addition of small amounts of glycerol decreases MSD(L) at short time scales. Note, however, that the faster decay of L-G HBs at longer time scales - and at 300 K - agrees with the plasticizing effect of glycerol usually reported in the literature, which is also revealed by the much faster decay of $C_{HB}$ for T-G HBs than for T-T ones at long time scales in both LTG and TG matrices at 300 K (see Figure S16 in the Supporting Information). Moreover, the slowing down of the structural relaxation of T-T, T-G, and T-W HBs induced by the presence of lysozyme (Figure S16 in the Supporting Information) suggests that (i) lysozyme confines the motions of trehalose, glycerol, and water and, therefore, hinders their diffusion (see e.g. the MSDs of glycerol and water in Figure S6 in the Supporting Information), and that (ii) the preferential interaction of glycerol and water with lysozyme in comparison with trehalose makes them less likely to plasticize trehalose motions. In addition, Figure 10b-c confirms that L-W HBs are stronger than L-T and L-G ones, in line with the results from Figure 9f and with the MD study of Tarek and Tobias on RNase A in glycerol or in water which suggests that protein-water HBs relax more slowly than protein-glycerol ones at sub-ps time scales (see Figure 4a of ref. [76]). Similarly, T-W HBs appear stronger than T-T and T-G ones in both LTG and TG matrices (Figure S16 in the Supporting Information). These results seem in line with a recent thermodynamic analysis of HBs in pure water and in a 0.8 m trehalose/water binary solution by Sapir and Harries, who determined that the enthalpic contribution to the free energy of HB formation, $\Delta G$, is larger in T-W HBs (-3.6 ± 0.4 kJ.mol$^{-1}$) than in T-T ones (-2.8 ± 0.6 kJ.mol$^{-1}$) (note that $\Delta G$ was only very slightly more favorable for T-W HBs than for T-T ones, -3.5 ± 0.5 kJ.mol$^{-1}$ vs -3.4 ± 0.9 kJ.mol$^{-1}$ at 298 K). We can thus assume that the reduced MSDs of lysozyme and trehalose in the presence of water stems from the formation of strong L-W and/or T-W HBs. At 300 K and time scales longer than ~ 1-10 ps, L-T and T-T HBs relax much slower than L-G or T-G ones, and even more than L-W and T-W HBs, thereby explaining why the substitution of L-T HBs by L-W and L-G ones on one hand and of T-T HBs by T-W and T-G ones on the other hand (see Figure 7 and Tables S4 and S5 in the Supporting Information) leads to a plasticizing effect of glycerol and water on lysozyme and trehalose.
motions. Conversely, L-W (T-W) HBs relax more slowly than L-T (T-T) and L-G (T-G) ones up to the ns time scale at 100 K (Figure 10c and Figure S16c,f in the Supporting Information), owing to the reduced molecular mobility of water at such a low temperature (Figure S6 in the Supporting Information). Thus, the more numerous and/or stronger HBs formed by lysozyme and trehalose in the presence of water or glycerol rationalizes why the fast dynamics of proteins may be better suppressed in these solvents than in trehalose at low temperatures. Indeed, the structural relaxation of protein-water and protein-glycerol HBs at such temperatures becomes so slow that the breaking of HBs on the ps-ns time scale then primarily depends on the strength of the HBs formed rather than on the diffusion of solvent molecules. Therefore, our results clarify why glycerol and water may exhibit both plasticizing and antiplasticizing effects, depending on the temperature considered, and corroborate previous experimental ones from the literature. For example, they are in line with those of Nickels et al., which demonstrated that hydration water reduces protein fast motions at low temperatures, but enhances them at higher temperatures. The present results also fairly agree with those of Garcia-Sakai et al., which showed that the quasi-elastic intensity of 1:1 L/G and 1:0.5 L/D₂O samples is weaker than those of dry L and 1:1 L/T ones at 150 K. Finally, they are consistent with the neutron and light scattering results of Caliskan et al., which evidenced that glycerol is more efficient than trehalose to dampen the dynamics of lysozyme at temperatures below \( \sim 270 \) K, whereas trehalose is more effective at higher temperatures, as expected from its higher \( T_g \) (\( \sim 390 \) K for glycerol vs \( \sim 190 \) K for trehalose).
Figure 10: Time autocorrelation function, $C_{\text{HB}}$, of different types of HBs found in the LTG matrices: L-T and L-G HBs at $h = 0.0$ and $w_G = 0$ or $5\%$ at 300 K (a), L-T, L-G, and L-W HBs at $h = 0.15$ and $w_G = 20\%$ at 300 K (b) and at 100 K (c). The insets show zooms on the 0.05-0.1 ps time range, in which the decay of $C_{\text{HB}}$ strongly depends on the strength of the considered HBs. The $C_{\text{HB}}$ of T-T, T-G, and T-W HBs in LTG and TG matrices are provided in Figure S16 in the Supporting Information.
Conclusions

This study evidences that glycerol and water at low concentrations modify the structural and dynamical properties of LTG and TG matrices to various extents. The addition of small amounts of glycerol and/or water decreases their glass transition temperature, $T_g$, and improves their molecular packing. However, the density of LTG matrices exhibit small maxima at glycerol contents of 5-10 wt. %, whereas that of TG mixtures steadily decreases when $w_G$ increases. This difference may be ascribed to the much larger excluded volume effects induced by the presence of lysozyme in LTG matrices, which prevents an efficient packing between protein and sugar molecules, given its large size, globular shape, and the roughness of its surface. Accordingly, LTG matrices have lower densities, larger free volume fractions and are composed of bigger holes than TG ones, and trehalose molecules occupy a larger volume in LTG matrices. Such differences modulate the effects of glycerol and water on the fast dynamics of lysozyme and trehalose, which depend on the time scale and the temperature considered. At 300 K, glycerol tends to act as an antiplasticizer on lysozyme and trehalose motions in LTG matrices by reducing their amplitudes at the ps and the ns time scales, but it mostly plasticizes trehalose motions in TG matrices. Water antiplasticizes the ps-time scale motions of lysozyme and trehalose in LTG and TG matrices (only at $h = 0.075$ in TG mixtures), and it exhibits a plasticizing effect at the ns time scale in both matrices. At 100 K, water systematically antiplasticizes the fast motions of lysozyme and trehalose in both LTG and TG systems, whatever the time scale (ps or ns) considered, and glycerol also shows such an effect at $h = 0.0$ and 0.075. The crossovers from a plasticizing effect at room temperature to an antiplasticizing one at lower temperatures that we found for water and glycerol at the ns time scale are consistent with those reported in previous experimental studies for glycerol in TG mixtures and for glycerol and water in protein matrices.

To understand the origin and the temperature dependence of those effects, we characterized the intermolecular HBs formed between species. Lysozyme and trehalose form more HBs in presence of glycerol and/or water. Nonetheless, the HBs that lysozyme forms with trehalose, glycerol, and water in LTG matrices are much less numerous that the ones it forms with its hydration water in dilute aqueous solution. Therefore, the water replacement hypothesis is only partially valid, since trehalose and glycerol cannot perfectly substitute for water upon dehydration. In addition, the distribution between species of the intermolecular HBs formed by lysozyme reveals that trehalose, glycerol, and water are not distributed homogeneously in the LTG mixtures. Rather, lysozyme interacts preferentially with water, even more when the water content decreases, in line with the water anchorage hypothesis, which assumes that residual water anchors the protein dynamics to its surroundings. Interestingly, our results also reveal that the preferential interaction of lysozyme with glycerol rather than with trehalose in the anhydrous LTG matrices necessarily stems from the larger size of trehalose and its more complex topology with respect to glycerol, since the hydroxyl groups of trehalose and glycerol share the very same non-bonded interaction parameters in our simulations. Besides, lysozyme-water HBs are stronger than lysozyme-glycerol and lysozyme-trehalose ones, which can be ascribed to the smaller size of water with respect to glycerol and trehalose as well as to its more polar O-H bond that allow it to interact more favorably with the protein than glycerol and trehalose do. Moreover, lysozyme-water and lysozyme-glycerol HBs relax much faster than lysozyme-trehalose ones at 300 K and at time scales greater...
than $\sim 10$-100 ps, owing to the larger mobilities of water and glycerol compared to that of trehalose, thereby corroborating the plasticizing effects of water and glycerol usually reported in the literature. Nonetheless, L-W HBs, and to a lower extent, L-G HBs, relax more slowly than L-T ones at 100 K, at which the mobilities of water and glycerol are strongly reduced, so that the structural relaxation of HBs depends to a larger extent on the strength of the HBs formed. This explains why at low temperatures the MSD of hydrated proteins may be reduced with respect to that of dry proteins or why the MSD of proteins in trehalose glasses may exceed that of proteins in glycerol solvent, as revealed by several experimental studies. Our results thus rationalize why glycerol performs better than trehalose at low temperatures. Given that the stability of proteins in sugar glasses has been correlated with the inverse of the MSD of the matrix in neutron scattering experiments, our results clearly suggest that the addition of small amounts of glycerol and water may improve the stability of freeze-dried proteins by increasing the number and/or the strength of the HBs they form, thereby stiffening the glassy matrix and reducing protein motions that may lead to denaturation.

Overall, the structural properties of the LTG and TG matrices investigated in this work (density, free volume fraction, numbers and distributions of HBs, etc.) exhibit dependences on the concentrations of glycerol and water that appear rather similar at 300 K and at 100 K, in contrast to dynamical properties (MSDs, structural relaxation of HBs). Moreover, at a given weight content, water usually influences such properties to a deeper extent than glycerol does, owing to its smaller size and more favorable interactions with lysozyme and trehalose. Finally, this study suggests that the potentially stabilizing effects of low concentrations of glycerol and water on proteins may probably not be deciphered by considering only mixtures of excipients and water, since proteins, excipients, and water mutually influence each other.

**Supporting Information Available**

Full simulation details; compositions and densities of simulated systems; contents of $\alpha$-helices and $\beta$-sheets of lysozyme; procedure for the determination of the glass transition temperature; densities and free volume fractions at 100 K; spatial distributions of free volume; discussion on the hole filling mechanism; molecular volumes of lysozyme and trehalose; examples of MSDs of lysozyme, trehalose, glycerol, and water; correlation between MSD and density; $n_{\text{HB}}(L)$ and $n_{\text{HB}}(T)$ in LTG and TG matrices; decompositions of $n_{\text{HB}}(L)$, $n_{\text{HB}}(T)$, $n_{\text{HB}}(G)$, and $n_{\text{HB}}(W)$; bridging glycerol and water molecules; geometry and dynamics of HBs in LTG and TG matrices.

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Supporting information for:
Molecular Packing, Hydrogen Bonding, and Fast Dynamics in Lysozyme/Trehalose/Glycerol and Trehalose/Glycerol Glasses at Low Hydration

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Simulation details

Simulation parameters

We performed molecular dynamics (MD) simulations using the CHARMM program,\textsuperscript{S1} version c35b1. Lysozyme molecules were represented using the all-atom CHARMM22 force field,\textsuperscript{S2} with the CMAP correction for backbone dihedral angles.\textsuperscript{S3} Trehalose and glycerol molecules were modeled with the CHARMM36 carbohydrate force field\textsuperscript{S4–S6} and the rigid SPC/E model\textsuperscript{S7} was considered for water. The covalent bonds involving an hydrogen atom and the geometry of water molecules were constrained using the SHAKE algorithm,\textsuperscript{S8} with a relative tolerance of $10^{-10}$. The equation of motion was integrated with the Verlet leapfrog algorithm\textsuperscript{S9} and a timestep of 1 fs. van der Waals interactions were smoothly force-switched\textsuperscript{S10} to zero between 8 and 10 Å, and Lorentz-Berthelot mixing-rules have been employed for cross-interaction terms. The particle mesh Ewald (PME) method\textsuperscript{S11} has been used to compute electrostatic interactions, with $\kappa = 0.33$ Å\textsuperscript{−1} and the fast-Fourier grid spacing set to approximately 1 Å. Any net translation and rotation of simulated systems was removed every 5 ps. The Langevin piston method\textsuperscript{S12,S13} was employed to control temperature and pressure during simulations. The mass of the thermal piston was set to 20000 kcal.mol\textsuperscript{−1}.ps\textsuperscript{2}. The mass of the pressure piston and the Langevin piston collision frequency were equal to 2000 amu and 20 ps\textsuperscript{−1}, respectively, for simulations in the isothermal-isobaric (N,P,T) ensemble. These last two parameters were set to zero for simulations in the canonical (N,V,T) ensemble. The charge state of ionizable amino acids corresponded to a pH of 7, and the total charge of lysozyme (+8 e) was neutralized by uniformly rescaling the charge of each protein atom, as performed in previous simulations.\textsuperscript{S14,S15} The initial conformation of trehalose molecules corresponded to that in the dihydrate crystal.\textsuperscript{S16}

Preparation and simulation of LTG and TG matrices

Lysozyme/trehalose/glycerol (LTG) and trehalose/glycerol (TG) glasses were prepared from the starting structures of the lysozyme/trehalose (LT) and trehalose (T) glasses from our previous study.\textsuperscript{S15} We first deleted successively trehalose molecules selected randomly, and then inserted glycerol molecules with random positions and orientations, with distance criteria that prevented any overlap between glycerol and lysozyme or trehalose molecules. The hydrated systems were generated from the dry ones by inserting water molecules with random positions and orientations. Then, each system was equilibrated in a similar way as in our previous work;\textsuperscript{S15} they were first minimized with the steepest descent and conjugate gradient algorithms, heated up from 100 K to 300 K at a rate of 5 K/ps (temperature was increased in steps of 10 K every 20 ps), equilibrated for 100 ps in the (N,V,T) ensemble, and then in the (N,P,T) ensemble, first at atmospheric pressure for 1 ns, and then at a pressure of 2 kbar for another 1 ns. The latter simulation aimed at densifying the simulation boxes without denaturing lysozyme molecules and/or changing significantly the conformation of trehalose molecules. Next, a 200-ps-long simulation at 1 atm was performed to relax the systems.

Following this preparation procedure, each system was heated up from 300 K to a maximum temperature, $T_{\text{max}}$, at rate of 0.5 K/ps, equilibrated at $T_{\text{max}}$ for 3 ns, and then cooled down to 300 K at a rate of 0.05 K/ps (temperature was decreased in steps of 10 K every 200 ps). For most systems, $T_{\text{max}}$ was set to 700 K, but for a few hydrated systems we needeed to decrease $T_{\text{max}}$ to 650 K to limit water evaporation. During this equilibration stage, we applied a mass-weighted besfit harmonic potential of 1.0 kcal/mol/Å\textsuperscript{2} on the heavy backbone atoms of lysozyme molecules (C, C\textsubscript{α}, N, and O) to keep stable their conformations. We also determined the pucker parameters\textsuperscript{S17} of the glucose rings of trehalose molecules at the end of the equilibration
procedure. Usually, a few of them deviated from the \( ^4C_1 \) chair conformation owing to the high temperatures reached. In such a case, we performed \((N,P,T)\) simulations of a few ps with a bestfit harmonic potential on the corresponding ring atoms until they went back to the chair conformation. We then run an additional equilibration simulation at 300 K and 1 atm for 3-70 ns to check for volume stabilization. Next, we performed simulations at 300 K in the \((N,V,T)\) ensemble for 25 ns at the equilibrated volume. We then cooled the final configurations from 300 K down to 100 K at a rate of 0.05 K/ps in the \((N,P,T)\) ensemble, performed an additional equilibration at 100 K for 2 ns, and subsequently run simulations at 100 K in the \((N,V,T)\) ensemble for 5 ns at the equilibrated volume. The last configuration of these simulations was then cooled down to 50 K at a rate of 0.05 K/ps, equilibrated for 1 ns at 50 K, and heated up to 800 K for LTG systems and to 700 K for TG ones, at a rate of 0.05 K/ps. The latter simulations were performed to estimate the glass transition temperature \( T_g \) of mixtures from the temperature dependence of their density. Configurations were saved every 0.2 ps for the analysis of production simulations. Additional short simulations (10 ps-long, with configurations saved every 0.002 ps) were also performed for the analysis of sub-ps dynamical properties. The compositions of the LTG and TG matrices and their densities at 300 K and at 100 K are given in Table S1. Furthermore, we determined the average contents of secondary structure (\( \alpha \)-helices and \( \beta \)-sheets) of lysozyme molecules in the LTG mixtures (see Table S2) and checked that their conformations at these temperatures remain close to their native one.

Table S1: Compositions and densities, \( \rho \), of the different simulated matrices as a function of the glycerol content, \( w_G \), and the hydration level, \( h \). \( w_G \) corresponds to the weight fraction of glycerol (in %) with respect to the total weight of excipients, and \( h \) refers to the weight fraction of water with respect to either lysozyme in the LTG glasses, or to trehalose and glycerol in the TG mixtures. \( N_L \), \( N_T \), \( N_G \) and \( N_W \) denote the numbers of lysozyme, trehalose, glycerol and water molecules, respectively. The densities \( \rho \) are given at the two temperatures considered in this study, 300 K and 100 K.

| \( h \) | \( \frac{g(H_2O)}{g(L \ or \ T+G)} \) | \( w_G \) (wt %) | LTG | TG | \( \rho \) (g.cm\(^{-3}\)) | \( \rho \) (g.cm\(^{-3}\)) |
|---|---|---|---|---|---|---|
| | | | \( N_L \) | \( N_T \) | \( N_G \) | \( N_W \) | \( T_g \) | \( N_L \) | \( N_T \) | \( N_G \) | \( N_W \) |
| 0.0 | 0 | 8 | 334 | 0 | 0 | 1.297 | 1.334 | 0 | 344 | 0 | 0 | 1.395 | 1.437 |
| | 5 | 8 | 317 | 62 | 0 | 1.302 | 1.338 | 0 | 317 | 62 | 0 | 1.392 | 1.432 |
| | 10 | 8 | 301 | 124 | 0 | 1.298 | 1.334 | 0 | 301 | 124 | 0 | 1.384 | 1.431 |
| | 15 | 8 | 284 | 186 | 0 | 1.295 | 1.331 | 0 | 284 | 186 | 0 | 1.376 | 1.420 |
| | 20 | 8 | 267 | 248 | 0 | 1.292 | 1.331 | 0 | 267 | 248 | 0 | 1.365 | 1.415 |
| 0.075 | 0 | 8 | 334 | 0 | 477 | 1.307 | 1.349 | 0 | 334 | 0 | 477 | 1.397 | 1.441 |
| | 5 | 8 | 317 | 62 | 477 | 1.306 | 1.345 | 0 | 317 | 62 | 477 | 1.386 | 1.433 |
| | 10 | 8 | 301 | 124 | 477 | 1.308 | 1.350 | 0 | 301 | 124 | 477 | 1.379 | 1.430 |
| | 15 | 8 | 284 | 186 | 477 | 1.304 | 1.344 | 0 | 284 | 186 | 477 | 1.373 | 1.422 |
| | 20 | 8 | 267 | 248 | 477 | 1.299 | 1.342 | 0 | 267 | 248 | 477 | 1.362 | 1.415 |
| 0.15 | 0 | 8 | 334 | 0 | 954 | 1.307 | 1.351 | 0 | 334 | 0 | 954 | 1.377 | 1.429 |
| | 5 | 8 | 317 | 62 | 954 | 1.309 | 1.352 | 0 | 317 | 62 | 954 | 1.367 | 1.420 |
| | 10 | 8 | 301 | 124 | 954 | 1.309 | 1.352 | 0 | 301 | 124 | 954 | 1.360 | 1.414 |
| | 15 | 8 | 284 | 186 | 954 | 1.307 | 1.351 | 0 | 284 | 186 | 954 | 1.351 | 1.405 |
| | 20 | 8 | 267 | 248 | 954 | 1.304 | 1.347 | 0 | 267 | 248 | 954 | 1.343 | 1.396 |
Table S2: Average $\alpha$-helix and $\beta$-sheet contents of lysozymes in the LTG glasses at 300 K and at 100 K determined with the Stride algorithm\textsuperscript{S19} (standard deviations are given in parentheses). The corresponding values for the crystalline conformation of lysozyme (193L PDB entry)\textsuperscript{S18} are 33 % and 6 %, respectively.

| Temperature (K) | w$_G$ (wt %) | $h$ [g(H$_2$O)/g(L)] | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ |
|----------------|--------------|-----------------------|---------|--------|---------|--------|---------|--------|
|                |              | 0.0                   | 0.075   | 0.15   | 0.0     | 0.075  | 0.15   |
| 300            | 0            | 28.3 (0.9)            | 4.8 (0.4) | 30.5 (0.6) | 6.7 (0.3) | 31.0 (0.8) | 6.6 (0.6) |
|                | 5            | 30.4 (0.9)            | 6.2 (0.5) | 31.1 (1.0) | 6.1 (0.6) | 30.9 (0.7) | 6.3 (0.5) |
|                | 10           | 30.4 (0.8)            | 6.0 (0.4) | 31.5 (0.8) | 6.3 (0.4) | 32.6 (0.9) | 5.9 (0.4) |
|                | 15           | 31.9 (0.8)            | 6.2 (0.4) | 31.9 (0.7) | 5.2 (0.5) | 33.0 (0.8) | 6.9 (0.4) |
|                | 20           | 30.6 (1.0)            | 5.8 (0.5) | 33.3 (1.0) | 6.2 (0.4) | 32.4 (0.9) | 7.7 (0.5) |
| 100            | 0            | 29.4 (0.5)            | 4.7 (0.3) | 30.9 (0.4) | 6.8 (0.2) | 31.7 (0.5) | 6.3 (0.5) |
|                | 5            | 31.6 (0.6)            | 6.5 (0.4) | 33.8 (0.5) | 6.8 (0.3) | 31.3 (0.6) | 6.7 (0.3) |
|                | 10           | 31.3 (0.6)            | 5.9 (0.3) | 32.9 (0.5) | 6.8 (0.3) | 34.2 (0.5) | 6.2 (0.3) |
|                | 15           | 32.4 (0.5)            | 6.7 (0.2) | 31.9 (0.4) | 5.4 (0.3) | 34.7 (0.5) | 7.0 (0.2) |
|                | 20           | 32.1 (0.6)            | 5.8 (0.3) | 35.1 (0.5) | 6.5 (0.2) | 33.6 (0.5) | 7.7 (0.3) |
Determination of the glass transition temperature

As a means to estimate the glass transition temperature, $T_g$, of the simulated LTG and TG systems, we determined the temperature dependence of their density, $\rho$, upon heating at a rate of 0.05 K/ps (Figure S1). The temperature dependence of $\rho$ was then fitted with an equation similar to that used in Averett et al.:

$$\rho(T) = \rho(T_g) + m_0(T_g - T) + 0.5 \alpha \log \left( 2 \cosh \left( \frac{T - T_g}{\delta T} \right) \right) + T - T_g$$

(1)

where $T$ is temperature, $m_0$ denotes the slope in the glassy regime, $\alpha$ characterizes the change of slope between the glassy and the rubbery regimes, which occurs over a temperature range of approximately $2 \times \delta T$. Given that the change of slope of $\rho$ for LTG systems occurs over a very broad temperature range (Figure S1a,c,e), we kept $\delta T$ fixed to values of 200 K for $h = 0.0$ and of 150 K for $h = 0.075$ and 0.15 for these systems (such values roughly correspond to the averages obtained when keeping free the $\delta T$ parameter during the fitting procedure, at a given hydration level, $h$). Moreover, the maximum temperatures considered for the fits were 800 K, 750 K, and 700 K for LTG matrices, and 700 K, 600 K, and 550 K for TG ones, at hydration levels, $h$, of 0.0, 0.075, and 0.15, respectively. Examples of fits are provided in Figure S2.
Figure S1: Temperature dependence of the density, \( \rho \), of LTG (left panel) and TG mixtures (right panel) upon heating at a rate of 0.05 K/ps: (a-b) \( h = 0.0 \), (c-d) \( h = 0.075 \), and (e-f) \( h = 0.15 \). The arrows illustrate the low-temperature shift induced by increasing the glycerol content, \( w_G \). The steep decreases of \( \rho \) for temperatures of \( \sim 800 \) K in LTG systems and of \( \sim 700 \) K in TG ones at \( h = 0.15 \) stem from water evaporation.
Figure S2: Examples of fits of the temperature dependence of the density, $\rho$, for LTG (left panel) and TG systems (right panel) at $w_G = 20\%$ and hydration levels, $h$, of 0.0 (a-b) or 0.15 (c-d) using equation (1). Error bars correspond to standard deviations of density fluctuations over the second half (100-200 ps) of heating simulations.
Molecular packing

Density-free volume fraction

Figure S3: (a-b) Density, ρ, and (c-d) free volume fraction, f, of the LTG and TG glasses as a function of the glycerol content, w_G, and the hydration level, h, at 100 K. f denotes the ratio of the total free volume accessible to a probe radius of 0.5 Å, V_f, to the total volume of the simulation box (f = 100*V_f/V).
Spatial distribution of free volume

Figure S4: Spatial distributions of free volume accessible to a probe radius of 1.0 Å in representative configurations of the LTG (top panels) and TG matrices (bottom panels) at various water and glycerol contents ($h = 0.0$, 0.075, and 0.15 and $w_G = 0$, 5, 20 %, respectively) and at 300 K. For clarity, only the holes whose volume is larger than about 15 and 5 Å³ are displayed for the LTG and TG matrices, respectively. This figure was generated using VMD (http://www.ks.uiuc.edu/Research/vmd/).
**Hole filling mechanism**

If we consider a glass whose volume is $V$ and which is composed of an homogeneous region of density $\rho$ and $N$ holes of volume $v$ (see Figure S5), then the free volume fraction, $f_0$, would be equal to $N.v/V$, and the corresponding density, $\rho_0$, would be equal to $\rho.(1-f_0)$. If we further assume that each of the free holes can host one filling molecule (water or glycerol for example) and that these molecules do not change the molecular structure of the glass, so that $V$ remains constant, then adding $n$ molecules ($n = \alpha.N$, where $0 \leq \alpha \leq 1$) would reduce the free volume fraction to $(1-\alpha).f_0$ and increase the density to $\rho_0+\alpha.f_0.\rho_1$, where $\rho_1$ is the density of a filled hole. If such hypotheses were true for the glasses investigated, then adding about 90 water molecules would be necessary to account for the decrease of $f$ for the LTG matrix at $w_G = 0$ % when $h$ raises from 0.0 to 0.075 (from 7.7 to 6.8 %, see Figure 2d), which is much lower than the actual number of water molecules added in the simulation box (477, see Table S1). Moreover, adding about 100 water molecules would be enough to explain the corresponding density increase (+ 0.01 g.cm$^{-3}$, see Figure 2a and Table S1). Similar observations can also be made for glycerol. Furthermore, Figure 3 indicates that only a small fraction number of holes (systematically lower than 0.05) would be large enough to host water molecules, assuming that they occupy the same volume in the glasses than in the bulk ($\sim 30$ Å$^3$ at a density of 1.0 g.cm$^{-3}$). Therefore, our results suggest that water and glycerol do not enhance the molecular packing of LTG and TG glasses by simply filling holes.

![Figure S5](image_url)

*Figure S5: Schematic representation of the hypothetic hole filling mechanism for a glass composed of holes of identical volume and whose structure does not change upon addition of filling molecules. $\alpha$ denotes the number fraction of holes filled by small molecules ($0 \leq \alpha \leq 1$).*
Molecular volumes

Table S3: Average molecular volumes, $\bar{V}$ (in Å$^3$), of lysozyme and trehalose in the LTG and TG glasses at 300 K. $\bar{V}$ were computed using Voronoi tessellation as performed by the program of Gerstein et al.$^{23}$ Standard deviations around mean values are given in parentheses.

| System | $w_G$ (%) | $h$ [g(H$_2$O)/g(L or T+G)] | 0.0     | 0.075    | 0.15     |
|--------|---------|-----------------------------|---------|----------|----------|
|        |         |                              | 0.0     | 0.075    | 0.15     |
| Lysozyme |         |                              |         |          |          |
|        | 0       | 18940 (145)                 | 18506 (166) | 18448 (217) |
|        | 5       | 18736 (149)                 | 18492 (149) | 18315 (130) |
| LTG    | 10      | 18736 (178)                 | 18461 (162) | 18236 (129) |
|        | 15      | 18723 (113)                 | 18434 (148) | 18196 (165) |
|        | 20      | 18723 (243)                 | 18409 (166) | 18210 (142) |
| Trehalose |        |                              |         |          |          |
|        | 0       | 424 (20)                    | 415 (17)  | 404 (16)  |
|        | 5       | 422 (20)                    | 412 (16)  | 403 (16)  |
| LTG    | 10      | 420 (20)                    | 407 (17)  | 401 (15)  |
|        | 15      | 419 (22)                    | 408 (16)  | 400 (14)  |
|        | 20      | 418 (17)                    | 408 (17)  | 398 (14)  |
| TG     | 10      | 404 (13)                    | 394 (13)  | 389 (12)  |
|        | 15      | 402 (14)                    | 391 (12)  | 388 (11)  |
|        | 20      | 402 (13)                    | 391 (12)  | 387 (11)  |
Mean-square displacements

Mean-square displacements of species

Figure S6: Time dependence of the mean-square displacement (MSD) of the hydrogens of lysozyme (L), trehalose (T), glycerol (G), and water (W) in LTG (left panel) and TG (right panel) matrices with $w_G = 10\%$ and $h = 0.15$: (a-b) at 300 K, (c-d) at 100 K.
Correlation between mean-square displacement and density

We checked whether changes in the mean-square displacement of LTG and TG matrices induced by the addition of glycerol or water are linearly correlated to changes in density. For this purpose, we assumed that the MSD at a given glycerol or water content, \( x (x = w_G \text{ or } h) \), may be written as:

\[
MSD(x) = MSD(0) + \alpha \cdot (\rho(x) - \rho(0))
\]  

where \( MSD(0) \) and \( \rho(0) \) correspond to the MSD and density in the absence of the considered diluent, and \( \alpha \) represents the constant that minimizes the following sum:

\[
\sum_x \left[ MSD - (MSD(0) + \alpha \cdot (\rho(x) - \rho(0))) \right]^2
\]

where MSD denotes the mean-square displacement of LTG and TG matrices determined directly from MD simulations, and the sum is over the several diluent contents, \( x \), considered in this study (\( w_G = 0, 5, 10, 15, 20 \% \) or \( h = 0.0, 0.075, 0.15 \)). Figures S7 and S8 show the MSDs of the LTG and TG systems as a function of \( w_G \) and \( h \), respectively, as well as the corresponding MSDs estimated from densities using eq 2. Although very simple, this phenomenological equation qualitatively predicts MSDs for both LTG and TG matrices at 300 K and at the ps time scale (Figure S7a,e). In particular, the maxima in the density of LTG matrices at \( w_G = 5 \) and 10 \% for \( h = 0.0 \) and 0.075, respectively, (Figure 2a) actually correspond to minima in MSDs (Figure S7a). Similarly, the decreases in density of TG matrices with \( w_G \) (Figure 2b) appear correlated to increases in MSD (Figure S7e,f). Furthermore, it remains difficult to evaluate the relevance of eq 2 for the addition of water, owing to the limited number of water contents, \( h \), considered in this study (0.0, 0.075, and 0.15). Yet, eq 2 seems to describe rather satisfactorily the change of MSD with \( h \) for some of the simulated systems (especially the LTG matrices with \( w_G = 5 \) and 20 \%, Figure S8). The antiplasticizing effect of water on LTG matrices at the ps time scale at 100 K is also well accounted for by the corresponding increase in density (Figure S8c). A much deeper analysis would be needed to explain on theoretical grounds how MSD relates to \( \rho \) in such complex systems. Nevertheless, Figures S7 and S8 suggest that \( \rho \) should not be overlooked when investigating antiplasticizing effects on the fast dynamics of glassy matrices.
Figure S7: Comparison of the mean-square displacements (MSDs) of hydrogens determined directly from simulations (full symbols) with those determined from eq 2 (empty symbols), as a function of the glycerol content, $w_G$, and for the different hydration levels, $h$. (a-d): LTG glasses, (e-h): TG ones. MSDs were averaged over the hydrogens from all species present in the LTG and TG systems. Error bars are not shown for clarity.
Figure S8: Comparison of the mean-square displacements (MSDs) of hydrogens determined directly from simulations (full symbols) with those determined from eq 2 (empty symbols), as a function of the hydration level, \( h \), and for the different glycerol contents, \( w_G \). (a-d): LTG glasses, (e-h): TG ones. MSDs were averaged over the hydrogens from all species present in the LTG and TG systems. Error bars are not shown for clarity.
Hydrogen bonds

Numbers of HBs of lysozyme and trehalose

Figure S9: Total numbers of intermolecular HBs, $n_{HB}$, formed by lysozyme, $n_{HB}(L)$, and trehalose, $n_{HB}(T)$, in the LTG (a-d) and TG matrices (e-f) at 300 K (left panel) and 100 K (right panel). The scales of axes are identical in (c-f) to make easier the comparison between the $n_{HB}(T)$ in the LTG and TG matrices. The $n_{HB}(T)$ values in TG matrices at 100 K and $h = 0.0$ are in fair agreement with those determined by Magazu et al. in ref. [S24] and reported in (f).
**Table S4: Decomposition of the mean numbers of intermolecular HBs formed by lysozyme (L), trehalose (T), glycerol (G), and water (W) in the LTG matrices at 300 K and at 100 K as a function of the glycerol concentration, w_G, and the hydration level, h. Standard deviations around mean values are given in parentheses.**

### 300 K

| Species | w_G (wt %) | T-L | L-L | L-W | T-G | T-L | T-W | G-L | G-W | W-L | W-W |
|---------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lysozyme | 0.0 | 20.1 (1.0) | 18.5 (1.0) | 19.9 (1.1) | 118.4 (1.0) | 122.5 (1.0) | 101.6 (1.0) | 71.2 (0.7) | 113.0 (1.8) |
| 5 | 21.3 (1.0) | 19.5 (1.2) | 24.7 (1.4) | 149.2 (2.0) | 131.1 (1.8) | 95.1 (1.7) | 15.3 (0.7) | 11.2 (0.7) | 10.5 (0.6) | 76.3 (1.4) | 118.1 (1.9) |
| 10 | 27.0 (1.1) | 22.4 (0.9) | 21.0 (1.2) | 127.0 (1.7) | 108.6 (1.7) | 91.1 (1.8) | 26.3 (0.8) | 20.2 (0.9) | 18.7 (1.2) | 72.1 (1.4) | 116.6 (1.9) |
| 15 | 20.7 (0.9) | 22.7 (1.0) | 21.1 (1.1) | 121.7 (1.7) | 106.0 (1.6) | 85.8 (1.3) | 37.4 (1.0) | 36.5 (1.3) | 27.9 (1.1) | 66.3 (1.3) | 103.1 (1.7) |
| 20 | 22.9 (1.1) | 24.8 (1.0) | 18.4 (1.0) | 114.0 (1.6) | 90.0 (1.5) | 81.6 (1.8) | 53.7 (1.2) | 47.1 (1.2) | 37.5 (1.2) | 66.4 (1.3) | 114.4 (1.8) |

### 100 K

| Species | w_G (wt %) | T-L | L-L | L-W | T-G | T-L | T-W | G-L | G-W | W-L | W-W |
|---------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lysozyme | 0.0 | 2.07 (0.5) | 1.54 (0.4) | 1.51 (0.4) | 3.28 (0.6) | 3.24 (0.6) | 3.16 (0.5) | 0.27 (0.2) | 0.23 (0.1) | 0.21 (0.2) | 0.82 (0.2) | 1.38 (0.3) |
| 5 | 1.78 (0.4) | 1.50 (0.3) | 1.26 (0.3) | 3.43 (0.4) | 3.22 (0.4) | 3.21 (0.5) | 0.52 (0.2) | 0.40 (0.2) | 0.41 (0.2) | 0.76 (0.3) | 1.66 (0.6) |
| 10 | 1.70 (0.2) | 1.73 (0.2) | 1.32 (0.2) | 3.39 (0.3) | 2.75 (0.3) | 2.63 (0.3) | 0.86 (0.2) | 0.64 (0.2) | 0.59 (0.2) | 0.87 (0.1) | 1.49 (0.2) |
| 15 | 1.82 (0.2) | 1.60 (0.2) | 1.26 (0.2) | 2.89 (0.3) | 2.60 (0.2) | 2.50 (0.2) | 0.94 (0.2) | 0.74 (0.2) | 0.77 (0.2) | 0.98 (0.1) | 1.47 (0.2) |
| 20 | 1.14 (0.1) | 1.09 (0.1) | 1.00 (0.1) | 1.72 (0.1) | 1.58 (0.1) | 1.59 (0.1) | 0.31 (0.0) | 0.09 (0.0) | 0.09 (0.0) | 0.66 (0.1) | 1.13 (0.1) |

| Glycerol | 0.0 | 2.68 (0.1) | 1.92 (0.1) | 1.64 (0.1) | 3.45 (0.1) | 3.22 (0.1) | 3.16 (0.1) | 0.52 (0.2) | 0.40 (0.2) | 0.41 (0.2) | 0.76 (0.3) | 1.66 (0.6) |
| 5 | 1.34 (0.1) | 1.02 (0.1) | 1.51 (0.1) | 1.54 (0.1) | 1.42 (0.1) | 1.42 (0.1) | 0.11 (0.0) | 0.09 (0.0) | 0.09 (0.0) | 0.66 (0.1) | 1.13 (0.1) |
| 10 | 1.23 (0.1) | 1.01 (0.1) | 1.56 (0.1) | 1.37 (0.1) | 1.20 (0.1) | 1.20 (0.1) | 0.20 (0.0) | 0.22 (0.0) | 0.22 (0.0) | 0.65 (0.1) | 1.06 (0.2) |
| 15 | 1.15 (0.1) | 1.00 (0.1) | 1.47 (0.1) | 1.31 (0.1) | 1.20 (0.1) | 1.20 (0.1) | 0.34 (0.0) | 0.29 (0.0) | 0.29 (0.0) | 0.64 (0.1) | 1.07 (0.2) |
| 20 | 1.14 (0.1) | 0.99 (0.1) | 1.32 (0.1) | 1.19 (0.1) | 1.09 (0.1) | 1.09 (0.1) | 0.51 (0.1) | 0.38 (0.0) | 0.38 (0.0) | 0.66 (0.1) | 1.11 (0.1) |
Table S5: Decomposition of the mean numbers of intermolecular HBs formed by trehalose (T), glycerol (G), and water (W) in the TG matrices at 300 K and at 100 K as a function of the glycerol concentration, \( w_G \), and the hydration level, \( h \). Standard deviations around mean values are given in parentheses.

### 300 K

| Species | \( w_G \) (wt %) | T-T | T-G | T-W | T-T | T-G | T-W |
|---------|-----------------|-----|-----|-----|-----|-----|-----|
|         | 0.0             | 0.05| 0.15|     | 0.0 | 0.05| 0.15|
| Trehalose| 0.0             | 14.02 (0.11) | 11.50 (0.11) | 9.89 (0.12) | 1.02 (0.03) | 0.91 (0.03) | 0.72 (0.03) | 4.80 (0.05) | 6.23 (0.07) |
|         | 5.0             | 13.02 (0.11) | 10.74 (0.11) | 9.33 (0.13) | 2.07 (0.04) | 1.68 (0.04) | 1.42 (0.05) | 3.77 (0.05) | 6.86 (0.09) |
|         | 10.0            | 12.03 (0.11) | 10.16 (0.11) | 8.72 (0.12) | 3.01 (0.05) | 2.39 (0.05) | 2.14 (0.06) | 5.77 (0.06) | 5.94 (0.07) |
|         | 15.0            | 11.17 (0.11) | 9.43 (0.11) | 8.11 (0.12) | 3.89 (0.06) | 3.18 (0.06) | 2.81 (0.07) | 6.77 (0.06) | 5.94 (0.07) |
|         | 20.0            | 10.34 (0.11) | 8.78 (0.13) | 7.43 (0.12) | 3.61 (0.06) | 3.18 (0.06) | 2.81 (0.07) | 5.87 (0.09) | 5.87 (0.09) |

### 100 K

| Species | \( w_G \) (wt %) | T-T | T-G | T-W | T-T | T-G | T-W |
|---------|-----------------|-----|-----|-----|-----|-----|-----|
|         | 0.0             | 0.05| 0.15|     | 0.0 | 0.05| 0.15|
| Trehalose| 0.0             | 15.00 (0.07) | 12.41 (0.07) | 10.65 (0.06) | 0.47 (0.08) | 0.21 (0.06) | 0.25 (0.08) | 1.22 (0.11) | 2.19 (0.14) |
|         | 5.0             | 14.01 (0.07) | 11.53 (0.06) | 10.14 (0.06) | 0.63 (0.08) | 0.50 (0.07) | 0.61 (0.11) | 1.34 (0.07) | 2.08 (0.09) |
|         | 10.0            | 13.10 (0.07) | 10.77 (0.06) | 9.35 (0.06) | 1.10 (0.07) | 0.96 (0.07) | 0.80 (0.07) | 2.86 (0.06) | 2.01 (0.08) |
|         | 15.0            | 11.98 (0.07) | 10.10 (0.06) | 8.71 (0.06) | 1.46 (0.07) | 1.19 (0.09) | 0.97 (0.09) | 3.77 (0.09) | 2.16 (0.07) |
|         | 20.0            | 11.22 (0.07) | 9.44 (0.06) | 8.06 (0.06) | 1.98 (0.04) | 1.64 (0.03) | 1.56 (0.02) | 3.88 (0.02) | 6.44 (0.03) |

### 100 K

| Species | \( w_G \) (wt %) | T-T | T-G | T-W | T-T | T-G | T-W |
|---------|-----------------|-----|-----|-----|-----|-----|-----|
|         | 0.0             | 0.05| 0.15|     | 0.0 | 0.05| 0.15|
| Trehalose| 0.0             | 15.00 (0.07) | 12.41 (0.07) | 10.65 (0.06) | 0.47 (0.08) | 0.21 (0.06) | 0.25 (0.08) | 1.22 (0.11) | 2.19 (0.14) |
|         | 5.0             | 14.01 (0.07) | 11.53 (0.06) | 10.14 (0.06) | 0.63 (0.08) | 0.50 (0.07) | 0.61 (0.11) | 1.34 (0.07) | 2.08 (0.09) |
|         | 10.0            | 13.10 (0.07) | 10.77 (0.06) | 9.35 (0.06) | 1.10 (0.07) | 0.96 (0.07) | 0.80 (0.07) | 2.86 (0.06) | 2.01 (0.08) |
|         | 15.0            | 11.98 (0.07) | 10.10 (0.06) | 8.71 (0.06) | 1.46 (0.07) | 1.19 (0.09) | 0.97 (0.09) | 3.77 (0.09) | 2.16 (0.07) |
|         | 20.0            | 11.22 (0.07) | 9.44 (0.06) | 8.06 (0.06) | 1.98 (0.04) | 1.64 (0.03) | 1.56 (0.02) | 3.88 (0.02) | 6.44 (0.03) |
Bridging glycerol and water molecules

The preferential interaction of lysozyme with water and, to a lower extent, with glycerol rather than with trehalose implies that water and glycerol can form *bridges* between lysozyme and trehalose molecules (see Figure S10). Therefore, we determined the number fractions of glycerol and water molecules that form simultaneously HBs with both lysozyme and trehalose, $F_{L-G-T}$ and $F_{L-W-T}$ (Figure S11a-b). In the anhydrous LTG mixtures, close to 80% of glycerol molecules are shared between lysozyme and trehalose at $w_G = 5\%$, and this proportion lowers to $\sim 65\%$ for larger glycerol contents. This may explain why the MSD of lysozyme at 300 K is minimal at $w_G = 5\%$ in these systems (Figure 6a-b), since glycerol locates primarily at the protein-sugar interface and, thus, is likely to improve the protein-matrix coupling. As expected, $F_{L-G-T}$ decreases when $h$ increases, owing to the substitution of L-G and G-T HBs by L-W and G-W HBs, respectively (see Figure 7d-l and Table S4). However, $F_{L-G-T}$ remains larger than 0.45 whatever the water and glycerol contents considered, thereby indicating that a significant amount of glycerol remains located at the protein-matrix interface in all LTG matrices. Moreover, the comparison of Figure 7a with Figure S11c-d indicates that glycerol and water molecules shared between lysozyme and trehalose form HBs with lysozyme that account for up to $\sim 20\%$ at $h = 0.0$ and up to $\sim 30\%$ at $h = 0.075$ and 0.15 of the total $n_{HB}$ of lysozyme, which suggests that these molecules play a role in the improved protein-matrix coupling.

We also calculated the number fractions, $F_{T-G-T}$, of glycerol molecules that form simultaneously HBs with the two rings of a given trehalose molecule in LTG and TG matrices (Figures S12 and S13), since these glycerol molecules may contribute to decrease the MSD of trehalose, as hypothesized by Dirama and coworkers. Figure S13 shows that these molecules account for about 15-25% and 15-40% of the glycerol molecules in LTG and TG matrices, respectively (depending on the glycerol and water concentrations and on the temperature considered). The comparison of Figure S13 with Figure 6e-l evidences that there is no straightforward relationship between $F_{T-G-T}$ and the MSD of trehalose in our simulations. Thus, our results suggest that there is no peculiar HB pattern between trehalose and glycerol molecules that may explain the decrease of the fast dynamics of trehalose induced by glycerol.
Figure S10: Examples of configurations where glycerol and water molecules form simultaneously HBs with both lysozyme and trehalose in the LTG matrices at 300 K. Left panel: $w_G = 5\%$ and $h = 0.075$; Right panel: $w_G = 20\%$ and $h = 0.15$. Lysozyme, trehalose, glycerol, and water molecules are colored in yellow/grey, red, green, and blue, respectively. Intermolecular HBs are represented as dashed purple lines. For clarity, neighboring molecules are not shown. This figure was generated using VMD (http://www.ks.uiuc.edu/Research/vmd/).
Figure S11: Top panel: Average number fractions, $F_{L-X-T}$, of glycerol (X=G) and water (X=W) molecules which are shared between lysozyme and trehalose (i.e. they form simultaneously HBs with both of them) in the LTG matrices, as a function of the glycerol content, $w_G$, and the hydration level, $h$: (a) at 300 K, (b) at 100 K. Bottom panel: Average number of intermolecular HBs, $n_{HB\,shared}$, that the glycerol and/or water molecules shared between lysozyme and trehalose form with a given lysozyme molecule, as a function of the glycerol content, $w_G$, and the hydration level, $h$: (c) at 300 K, (d) at 100 K.

Figure S12: Examples of configurations where a glycerol molecule forms simultaneously HBs with the two rings of a given trehalose molecule at 300 K. Left: in the TG mixture with $w_G = 5\%$ and $h = 0.0$. Right: in the LTG mixture with $w_G = 5\%$ and $h = 0.0$. Intermolecular HBs are represented as dashed purple lines. For clarity, neighboring molecules are not shown. This figure was generated using VMD (http://www.ks.uiuc.edu/Research/vmd/).\textsuperscript{S22}
Figure S13: Average number fractions, $F_{T\cdot G\cdot T}$, of glycerol molecules that form HBs simultaneously with the two rings of trehalose molecules in the LTG (left panel) and TG matrices (right panel) as a function of the glycerol content, $w_G$, and the hydration level, $h$: (a-b) at 300 K, (c-d) at 100 K.
Geometry of HBs

Figure S14: Distributions of the donor-acceptor distance (left panel) and donor-hydrogen-acceptor angle (right panel) of various HBs formed in the LTG matrices at 100 K: (a-b) L-T and L-G HBs at $h = 0.0$ and $w_G = 5\%$, (c-d) L-G HBs at $h = 0.0$ and $w_G = 5, 10, 15, \text{ and } 20\%$, (e-f) L-T, L-G, and L-W HBs at $h = 0.15$ and $w_G = 20\%$. 
Figure S15: Distributions of the donor-acceptor distance and donor-hydrogen-acceptor angle of various HBs formed in the TG matrices at 300 K (left panels) and at 100 K (right panels): (a-b) and (g-h) T-T and T-G HBs at $h = 0.0$ and $w_G = 5\%$, (c-d) and (i-j) T-T, T-G, and T-W HBs at $h = 0.15$ and $w_G = 5\%$, (e-f) and (k-l) T-T, T-G, and T-W HBs at $h = 0.15$ and $w_G = 20\%$. 
Figure S16: Time autocorrelation function, \( C_{HB} \), of various HBs involving trehalose in LTG (left panel) and TG matrices (right panel): T-T and T-G HBs at \( h = 0.0 \) and \( w_G = 0 \) or 5 % at 300 K (a,d), T-T, T-G, and T-W HBs at \( h = 0.15 \) and \( w_G = 20 \% \) at 300 K (b,e) and at 100 K (c,f). The insets show zooms on the 0.05-0.1 ps time range, in which the decay of \( C_{HB} \) strongly depends on the strength of the considered HBs.
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