Self-assembly of trehalose molecules on a lysozyme surface: the broken glass hypothesis

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To help understand how sugar interactions with proteins stabilise biomolecular structures, we compare the three main hypotheses for the phenomenon with the results of long molecular dynamics simulations on lysozyme in aqueous trehalose solution (0.75 M). We show that the *water replacement* and *water entrapment* hypotheses need not be mutually exclusive, because the trehalose molecules assemble in distinctive clusters on the surface of the protein. The flexibility of the protein backbone is reduced under the sugar patches supporting earlier findings that link reduced flexibility of the protein with its higher stability. The results explain the apparent contradiction between different experimental and theoretical results for trehalose effects on proteins.

1. Introduction

Sugar solutions can help biomolecules preserve their structure under harsh conditions, including dehydration and high temperatures. Among the naturally available disaccharides, trehalose appears to be the one of the most effective stabilising agents.[1–7] Trehalose, which is also called mycose and mushroom sugar,[1] is a nonreducing homodisaccharide in which two D-glucopyranose units are linked together in an α − 1, 1-glycosidic linkage.

Despite many experimental and theoretical studies on trehalose–protein interactions,[8–16] little is known about molecular mechanism of the trehalose stabilising effect on biomolecular structure because the experimental results are difficult to interpret on an atomistic level. It is reasonable to assume that stabilisation is a result of special interactions of the sugar molecules with the protein that lead to the formation of non-trivial, stabilising molecule structures. There are three main hypotheses describing such structures:[1]

- *Mechanical Entrapment (Vitrification) Hypothesis*: the entrapment of biomolecules in a glassy matrix of trehalose formed in high-viscosity *concentrated* trehalose solutions. This should protect the native conformation of biomolecules rather like insects trapped in amber.

- *Water Replacement Hypothesis*: protection of biomolecules through direct interaction between trehalose and the biomolecule surface groups through hydrogen bonding. This hypothesis suggests that most of water molecules in the first hydration shell of the biomolecule should be replaced by trehalose.

- *Water Entrapment Hypothesis*: trapping of water molecules in an intermediate layer between sugars and the biomolecular surface.

We note that the first hypothesis can only be applied to solutions with very high trehalose concentrations ($\gg$1 M) and low water content, as the system has to be virtually dry before the trehalose can form a glassy matrix. Trehalose appears to have particularly favorable properties under these conditions.[17] However, this does not explain why trehalose also shows good stabilisation properties at low and medium concentrated solutions ($\approx$0.05–1.0 M) which are natural for living organisms.[18–20]

Both *water replacement* and *water entrapment* hypotheses are supported by different sets of experimental and theoretical data. Infrared spectroscopy experiments[21] show that there is a large number of direct hydrogen bonds formed between trehalose and lysozyme, which is suggestive of the *water replacement hypothesis*. However, there is also much experimental[8–11] and theoretical[12,13,15,16] data showing that the protein-sugar interactions are better described in terms of *water entrapment* hypotheses.

The goal of this study is to revisit the question of the nature of trehalose–protein interactions and to reveal molecular-level details of binding of trehalose and water to proteins in medium-concentrated solutions by long-scale molecular dynamics simulations of lysozyme in aqueous trehalose solution (0.75 M).

Investigating particular molecular *structures* formed by interacting trehalose molecules with protein in water we have also found a distinctive effect of such structures on the *dynamics* of the protein. A clear correlation between the flexibility of the protein and the clusters of the sugar molecules has been found that may be related to the structural stability of the protein. That protein rigidity is a prerequisite for protein thermostability is a working hypothesis used by Vieille and Zeikus.[22]

Lysozyme folding and unfolding takes far longer than is currently possible for anyone to simulate if the necessary

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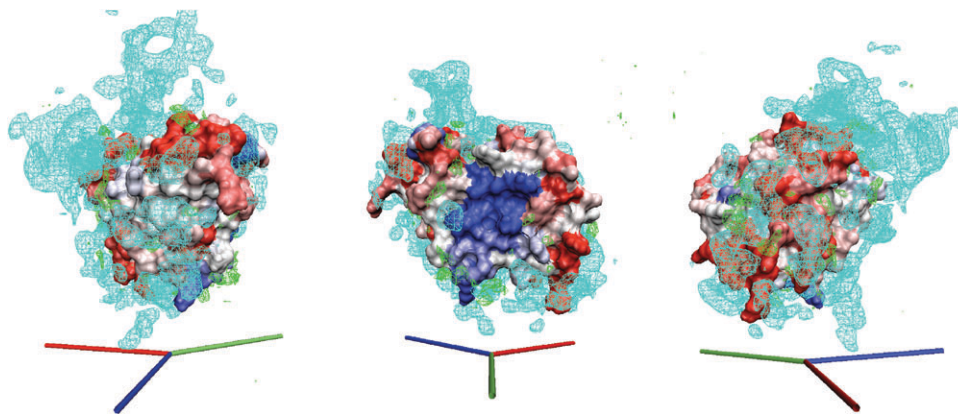
Fig. 1 Protein's surface coloured according to the difference in flexibility of the backbone caused by trehalose (the colours correspond to the difference plot shown in Fig. 3); cyan: trehalose density (6.4 times higher than in the bulk), see section 2 for definition; green: water density (1.5 times higher than bulk water) the molecular structure from three different viewpoints is shown.

number of water molecules and trehalose molecules are included.[23,24] It is possible, however, to analyse the flexibility of the lysozyme backbone atoms, and this should be related to the stability of the tertiary structure of the protein. This connection has been demonstrated for thermophilic proteins, which are biochemically active at high temperature. Experimental investigations have shown that this thermal stability is directly linked to flexibility of these proteins[25] and that the thermophilic proteins are as flexible at high temperature as mesophilic ones are at room temperature.[26] An NMR study on the effect of mannosylglycerate on staphylococcal nuclease shows that the mannosylglycerate reduced the backbone motion of the protein.[27] We expect, therefore, that the flexibility of lysozyme will be affected by the trehalose. If the mechanical entrapment hypothesis is correct, a reduction of flexibility should occur for the whole protein, whereas the two alternative hypotheses should lead to more localised effects.

2. Simulation details

We have performed 30 ns atomistic molecular dynamics (MD) simulations at room-temperature (300 K) of aqueous hen-egg lysozyme solution in the presence and absence of trehalose. We placed the protein into the centre of a cubic simulation box which also contained (i) $\sim 2.2 \times 10^4$ water molecules for the bulk water solution; (ii) $\sim 1.4 \times 10^4$ water molecules and 256 trehalose molecules for the sugar aqueous solution. The size of the box was adjusted using a one nanosecond simulation at a constant external pressure of one atmosphere. The simulations started from the conformation of the protein provided by the NMR data in the Protein Data Bank (PDB code 1e8l;[28] we took the 26th conformation from the 50 conformations provided by this PDB entry) using the GROMACS 3.3 molecular dynamics software. At the beginning of the simulation, trehalose molecules were randomly distributed throughout the system to give a concentration of 0.75 M. The initial distribution of trehalose molecules across the simulation box showed no clusters. Before the production runs, each system was equilibrated for 4 ns with the positions of the

oligopeptide atoms constrained. During this equilibration, the initial random distribution of trehalose molecules changed to form clusters on the protein surface which remained throughout the production runs. The solution was neutralised by addition of several counterions into the simulation cell. We used GROMOS 53a6 force-field[29] for the protein, ions and trehalose together with the SPC/E water model.[30] We used sugar parameters optimized for the GROMOS force field.[31] The force-field has been chosen for its adequate description of proteins as well as oligosaccharides.[29,31,32] The MD integration time-step was 2 fs, the electrostatic interactions were treated with the Reaction Field correction technique.[33]

To analyse the density of sugar and water in the system, the isodensity surfaces were computed as the number of atoms occupying three-dimensional grid points and averaged over 30 ns simulation (a grid point is considered occupied if it lies inside the sphere of the atomic radius centered on any atom of any sugar or water molecule). The occupancy number relative to the average number in the bulk is plotted in Fig. 1 and 2. The figure shows the volume where the sugar occupancy is 6.4 times higher than in the rest of the solution. This value was chosen because it gives a clear impression of the high sugar density around some parts of the protein. There are also some areas where water has more than 1.5 times the average occupation, because there is less sugar in these places, and this is also shown in Fig. 1. The electrostatic interactions were treated using the reaction field approach, as implemented in GROMACS 3.3.[34] For integration of the equations of motion we used the standard Verlet algorithm with a time step of 2.5 fs. The systems were coupled with a heat bath of 300 K temperature using a Berendsen thermostat[35] and the simulations took approximately 30 000 h of CPU time using 2 GHz AMD Opteron processors in a parallel cluster. After an equilibration period, the simulation ran with an approximately constant potential energy, demonstrating that there were no major changes in structure as the simulation progressed, and indicating that a reasonable level of convergence had been attained (Fig. 2, inset).
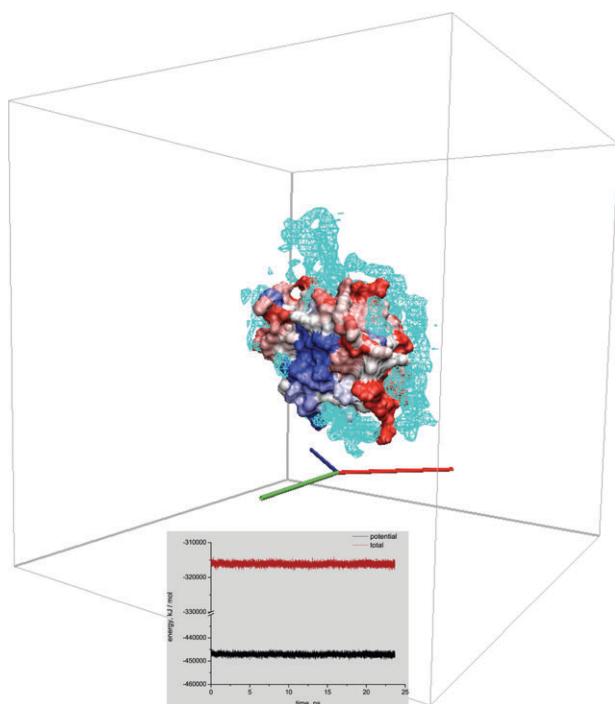
Fig. 2 Protein's surface coloured according to the difference in flexibility of the backbone caused by trehalose (the colours correspond to the difference plot shown in Fig. 3); cyan: trehalose density (6.4 times higher than in the bulk), see section 2 for definition; the simulation box is shown emphasising the absence of sugar everywhere except the vicinity of the protein. The inset shows the changes in potential energy and total energy as the simulation progressed.
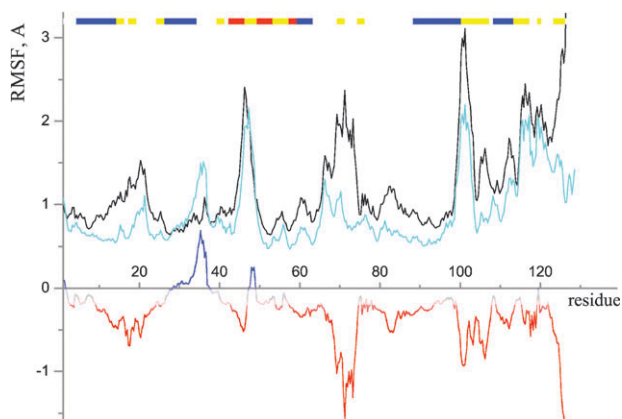

Fig. 3 RMSF of the protein's backbone atoms; black: 30 ns simulation in pure water solution; cyan: 30 ns simulation in trehalose-water solution; multi-colour line: difference in RMSF between water and trehalose solutions (cyan and black lines), the colours are used in mapping the difference to the protein's surface, Fig. 1 and 2 and protein's structure, Fig. 7; the assignment of the peptide's structural motifs for each amino acid is shown as a coloured strip above the curves: red - β-sheet, blue - α-helix, yellow - turn.

3. Results

During the course of the simulation, the protein stays in its folded state. The centre of mass and the principal axes of the protein were matched, and then the root-mean square

atomic position fluctuations (RMSF) were calculated for backbone atoms of the protein (carbon, oxygen and nitrogen). The average RMSF was calculated for each amino acid for the whole simulation. The distribution of the RMSF with respect to position along the backbone is shown in Fig. 3.

The analysis of available NMR experimental data[28] clearly shows that the flexibility pattern of the peptide is well reproduced in the simulation, Fig. 4. The larger values for RMSF of the MD simulation were expected, because the NMR derived structures were constrained by the experimental measurements.

When comparing the flexibility of the peptide in pure water and sugar solutions, a reduction of the motion of the backbone protein atoms in the trehalose solution is clearly seen, particularly at the C-terminus amino acids (residues 123–129) which are highly mobile in bulk water solution, Fig. 3. This non-uniform reduction of protein mobility cannot be attributed only to the increased viscosity of the sugar solution because this should only be twice as high for this sugar concentration as in the pure water solution.[36]

We note that amino acids 30–40 and 48–50 become more mobile with the addition of trehalose to the water solution. This increase in backbone mobility with trehalose occurs around the position of two active site amino acids GLU35 and ASP52 and Fig. 1 shows that this increase in flexibility is associated with the active site of the enzyme. Lysozymes specifically bind to peptidoglycans and oligosaccharides found in the cells walls of bacteria.[37–41] The enzymes hydrolyse glycosidic bonds in these molecules by distorting the ring into a half-chair conformation. In this strained state the glycosidic bond is easily broken.[38,39,41] Therefore, the active site amino acids of the hen-egg lysozyme should interact with sugars, but this interaction will be optimised for a hydrolysis process rather than tight binding. It is interesting to note that the active site correspond to the only positive peaks in Fig. 3.

In order to elucidate the mechanism of the trehalose influence we have plotted the sugar density with respect to the protein's surface, Fig. 1 and 2. Two important conclusions can be immediately drawn from the figures: (i) sugar molecules make clusters *only in the vicinity of the protein* (Fig. 2) and (ii) the cluster locations *correspond to the location of the reduced flexibility*. In addition, there are also small high-density water clusters on the surface of the lysozyme in
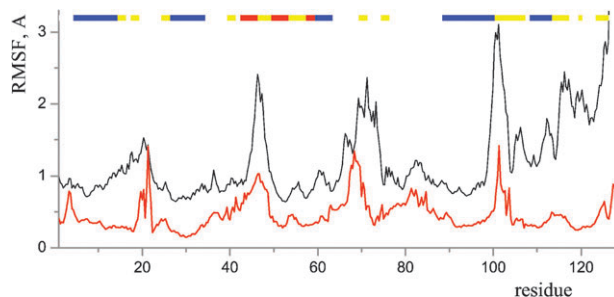

Fig. 4 RMSF of the protein's backbone atoms; black: 30 ns simulation in pure water solution; red: 50 NMR structures from ref. 28; the assignment of the peptide's structural motifs for each amino acid is shown as a coloured strip above the curves: red - β-sheet, blue - α-helix, yellow - turn.
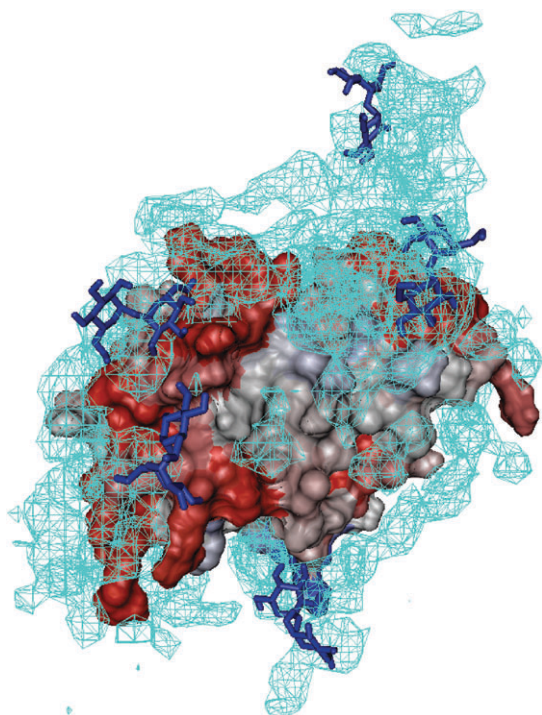
Fig. 5 Several randomly chosen sugar molecules shown to compare the size of the high density sugar patches with the size of the sugar molecules.

trehalose solution, and this is consistent with the *water entrapment hypothesis*. We note, that an increase of water density around lysozyme has been observed experimentally by X-ray and neutron scattering.[42] The size of the sugar clusters with respect to the individual trehalose molecules is illustrated in Fig. 5 in which some of the sugar molecules are drawn explicitly for a randomly chosen frame of the simulation.

We have calculated the number of protein-sugar hydrogen bonds, and the results are shown in Fig. 6 with the corresponding structural assignments in Fig. 7. The amino acids that form most hydrogen bonds with sugar either show reduced mobility or are located in the vicinity of the less mobile amino acids. Thus, our hypothesis is that hydrogen bonding (and, therefore, an immobilising effect) to the protein facilitates the formation of long lived sugar clusters that in turn reduces the flexibility of
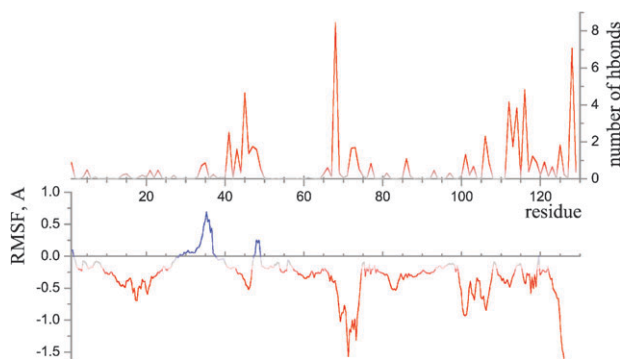

Fig. 6 Average number of lysozyme-trehalose hydrogen bonds per amino acid; the flexibility difference of the amino acids (same as in Fig. 3) is also shown.

the protein's backbone. This is consistent with the *water replacement hypothesis*.

To understand the general trends in the mechanism of protein binding with water and trehalose we calculated the average number of internal protein–protein hydrogen bonds for both water and trehalose solutions, and found that this is, on average, 10% less for the trehalose solution than for the pure water solution. Table 1 shows how many hydrogen bonds are formed by protein–trehalose and protein–water interactions. The results are shown only for those amino acids which side chains are able to form hydrogen bonds (charged, polar, and some aromatic amino acids). At the sugar concentration used in the study (0.75 M), trehalose has only a tenth of the hydrogen bonding sites of water. However, trehalose forms more than 20% of the total number of hydrogen bonds with the majority of the side chains. Moreover, trehalose interactions with acidic amino acids (GLU and ASP) are even more favourable—they form about 40% of the total hydrogen bonds to these amino acid side chains. This is a clear indication of preferential interactions of trehalose molecules with polar, and, especially, acidic amino acids.

4. Discussion

The flexibility of a protein backbone is connected to the stability of the tertiary structure, but the details of the molecular mechanism for this are not clear.[43] There is, however, some evidence that suggests a correlation between the flexibility of proteins and their structural stability.

NMR spin relaxation experiments have demonstrated that a chemical denaturant increases the fluctuations of the protein that eventually leads to its unfolding.[44] Interestingly, agents that stabilise proteins, like trimethylamine N-oxide (TMAO) used in the study, also reduce the flexibility of the protein.

In the same publication[44] it is concluded that TMAO does not bind specifically to the protein, suggesting a uniform stabilising effect. Similarly, molecular dynamics investigations[45] lead to a picture of lysozyme stabilisation by trehalose by the formation of a "glass" like substance. Raman scattering has also revealed the stabilisation effect of trehalose on lysozyme which remains folded at higher temperatures compared to pure water solutions.[46] The sugar effect becomes apparent starting from low sugar weight concentrations of as little as 20%. Both molecular dynamics[15] and experimental[47] results demonstrate the reduced fluctuations of the lysozyme in trehalose solutions.

Even though it is not possible to conclude with confidence that lower flexibility results in higher stability,[48] our finding adds more information in support of this hypothesis.

Our results demonstrate that the effect of the trehalose is non-uniform, and the interactions are specific for the areas between the secondary structural elements of the protein structure. There is one area where the protein becomes more flexible on the addition of trehalose, and this corresponds to the active site of the enzyme. The function of lysozyme is to hydrolyse glycosidic bonds, and the structure of trehalose is related to its natural substrates. Molecular dynamics simulations are not able to analyse chemical reactions, but the specific
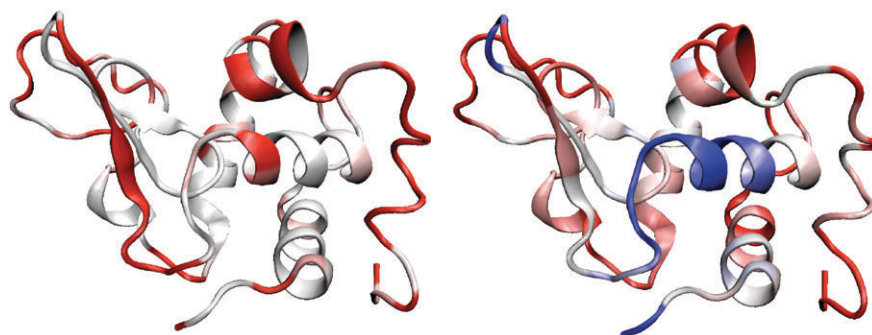
Fig. 7 Left: average hydrogen bonds per amino acid; right: flexibility of the amino acids (the colouring corresponds to Fig. 6).

Table 1 Hydrogen bonds (HBs) of the protein side-chains with trehalose and water as a percentage of the total number of amino acid-solvent HBs

Amino acid	Protein–trehalose	Protein–water
LYS	22	78
ARG	22	78
GLU	39	61
ASP	44	56
GLN	20	80
ASN	24	76
SER	26	74
THR	21	79
HIS	14	86
TYR	11	89
TRP	24	76

flexibility-increasing interaction of trehalose with the active site is suggestive of the catalytic function of the enzyme.

Very recently Sun has reported related calculations on chymotrypsin inhibitor 2 (CI2) for a lower concentration of trehalose and a much higher temperature (363 K).[49] This study concludes that CI2 stabilisation at this temperature is due to water entrapment. It may be that the difference between Sun's conclusion and ours is due to the differences in enzyme, concentration and temperature. It is also possible that the small average changes that Sun reports for water molecules around the protein can also be explained by the small discrete water clusters that are illustrated in Fig. 1.

5. Conclusions

We conclude that the trehalose distribution around the surface of lysozyme is non-uniform and the trehalose forms patches on the surface. Therefore, conclusions from experimental and theoretical studies which assume uniform distributions may be misleading. The results may be interpreted as providing support for both the water entrapment and the water replacement hypotheses; the structure of the trehalose patches is consistent with the latter, whereas the presence of water clusters adjacent to the sugar clusters is consistent with the former. The non-uniform distribution of the trehalose means that both hypotheses are valid, but for different parts of the structure. Because of the different chemical nature of the protein structural elements some (about 30%) of them prefer to interact directly with sugars. However, despite the large number of trehalose molecules near the protein surface, there

is plenty of room for water molecules too and most of the protein surface (about 70%) remains hydrated.

The trehalose clusters significantly reduce the mobility of the adjacent lysozyme amino acids except for a few amino acids close to the active site. Moreover, most of the clusters are concentrated around turns and the less structured elements of the protein. Therefore, this can be interpreted as trehalose having the greatest effect on the stability of the *tertiary* structure of the protein rather than on the *secondary* structural elements.

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