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CALORIMETRIC STUDY OF MYOGLOBIN EMBEDDED IN TREHALOSE–WATER MATRIXES

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It has been suggested that in 'dry' protein–trehalose–water systems, water–mediated hydrogen bond network, whose strength increases by drying, anchors the protein to its surroundings. To further characterize this effect, we performed a DSC study on low-water myoglobin–trehalose systems. The denaturation temperature resulted to increase by decreasing hydration, and linearly correlated to the glass transition temperature of both the ternary protein–water–trehalose and the binary water–trehalose systems. Further measurements are being performed to investigate eventual differences among different saccharides.

Keywords: *denaturation, DSC, glass transition, myoglobin, trehalose*

Introduction

Trehalose (α-*D*-glucopyranosyl-α-*D*-glucopyranoside) is a non-reducing disaccharide of glucose (Fig. 1). It is found in organisms that survive adverse environmental conditions such as extreme drought and high temperature [1]; furthermore, isolated structures as enzymes or liposomes are preserved against stressing conditions when embedded in trehalose matrixes [2, 3]. Biopreservation is also accomplished by other saccharides; however, trehalose results the most effective bioprotectant [3]. As other saccharides, trehalose is also a glass former [4–8].

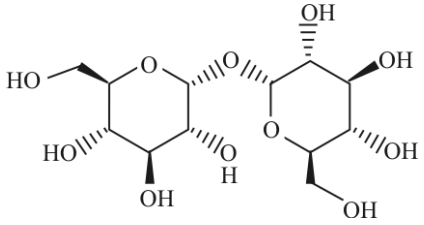

Fig. 1 The trehalose molecule

The main hypotheses suggested for explaining trehalose–biomolecule interaction are:

- water replacement hypothesis [9] according to which trehalose binds through H-bonds to the protein, thus replacing the first hydration shell;
- preferential hydration hypothesis (or water-entrapment) [10], according to which in the dry state, trehalose, rather than directly binding to protein, traps the residual water at the biomolecule sugar interface;
- high viscosity hypothesis [11], according to which viscosity effects cause motional inhibition and hindering of the processes which lead to loss of structure and denaturation. In particular it has been reported that solvent viscosity, is responsible for the reduction of anharmonic motions and for the slowing down of conformational relaxations of proteins encapsulated in silica hydrogels [12–14]. Furthermore, Green and Angell [4] suggested the rather high glass transition temperature of trehalose with respect to other glass forming sugars to be responsible for the trehalose peculiarity.

The hypotheses just illustrated are not mutually exclusive. Several simulative and experimental results suggest that preferential hydration better describes the interaction between globular proteins and sugars [15–18], while water replacement seems to hold true in membrane–sugar–water systems [2, 19–22]. Furthermore, trehalose peculiarity can be related to its glass forming and H-bonding properties: several simulative and experimental works point out that trehalose sizably modifies H-bond networks and water dynamics in binary water–sugar systems [23–25].

Recently a model has been proposed [26–28] that explains the trehalose peculiarity based on the outcome of some molecular dynamics (MD) simulations [15, 29] and FTIR experiments [18, 30]. In particular, simulations of a MbCO–trehalose–water system at 50% trehalose mass fraction (~20 water/sugar molar ratio) showed that the protein exchanges either single or multiple hydrogen bonds (HBs) with water, while, essentially, single OH groups of few trehalose molecules are hydrogen bonded to the protein through

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a single (donor) HB. The behaviour of the 50% system was found to extend to a drier system at 89% trehalose mass fraction (~2.4 water/sugar molar ratio), where a larger number of trehalose molecules were found bound to the protein, still through only one of their eight OH groups, again forming only a single hydrogen bond [15]. Further information on the protein–sugar–water structures present in the systems, were obtained from the analysis of the water molecules hydrogen bonded both to the protein and to the sugar OH groups. In the water-rich system, only 40% of the overall number of molecules hydrogen bonded to the protein was found also bound to trehalose, while in the drier system such a fraction increases to 73%. The above MD results, analysed on the light of FTIR measurements on MbCO embedded in trehalose matrixes, enabled to infer the existence of a water-mediated HB network, whose strength increases by decreasing the sample water content, which couples the internal degrees of freedom of the protein to those of the water–trehalose matrix [26]. Similar results were also obtained for MbCO embedded in low-water systems of other saccharides (sucrose, maltose, raffinose and glucose) [18]: a comparison showed that the coupling is the tightest for trehalose. Furthermore, in a very recent paper it has been shown that for the reaction centre of Rhodobacter Sphaeroides the protein–matrix coupling, which is very tight in trehalose, is absent in sucrose [27].

In order to realize how the above protein–matrix interactions are reflected on the thermodynamic properties of the system, we performed differential scanning calorimetry (DSC) measurements in which we studied ferric myoglobin (Mb) embedded in low water trehalose matrixes. In particular, we measured the matrix glass transition and myoglobin thermal denaturation in the ternary systems; furthermore, we analysed the results obtained in the light of results obtained in binary trehalose–water systems.

Experimental

Lyophilized ferric horse myoglobin was purchased from Sigma (Sigma, St. Louis, MO) and used without further purification. Trehalose (α-*D*-glucopyranosyl-α-*D*-glucopyranoside) from Hayashibara Shoij (Hayashibara Shoij Inc., Okayama, Japan) was used after recrystallization from aqueous solutions. For sample preparation, myoglobin was dissolved ($5\cdot10^{-3}$ M) in a solution containing $2\cdot10^{-1}$ M trehalose and $2\cdot10^{-2}$ M phosphate buffer (pH 7 in water). Aliquot (20 μL) of the above solution was deposited in an aluminium pan for volatile sample with a maximum volume of 20 μL and a mass of about 23 mg. Then the sample was blow-dried at 328 K until achieving the suitable water/sugar ratio. The water content was estimated by weighing the sample before and after blow-drying. Further depositions of solution and blow-drying were performed until reaching water/sugar ratios spanning from ~300 to ~2 water/sugar molar ratio and a myoglobin content ~5 mg. Aluminium pans were then sealed.

Calorimetric measurements were performed by a Diamond DSC PerkinElmer® with a Cryofill device. Indium was used to calibrate temperature and heat flow. Heat flow error is 0.05 mW. The temperature program consisted of two identical cycles performed as follows: cooling from 303 to 95 K at 500 K min^{-1}, holding 3 min, then warming to 393 K at 10 K min^{-1}. An empty sealed pan was used as a reference. In order to check the baseline stability and to match the aluminium mass contribution, a temperature cycle on a second empty pan was performed after each measurement.

The specific heat was evaluated by subtracting the aluminium heat flow normalized to its mass, then dividing by the scan rate and the sample mass. The temperature of the irreversible endothermic peak, related to myoglobin denaturation, was considered as the denaturation temperature (T_{den}). The denaturation enthalpy (ΔH_{den}) was estimated by the area under the denaturation peak, after subtraction of a suitable cubic polynomial curve [31]. The temperature value at the onset of the leap of specific heat, at low temperature, characterized the glass transition temperature (T_g).

Results and discussion

Typical upscan curves of protein–trehalose–water systems at different water/sugar mole ratios are shown in Fig. 2. The specific heat leap due to the

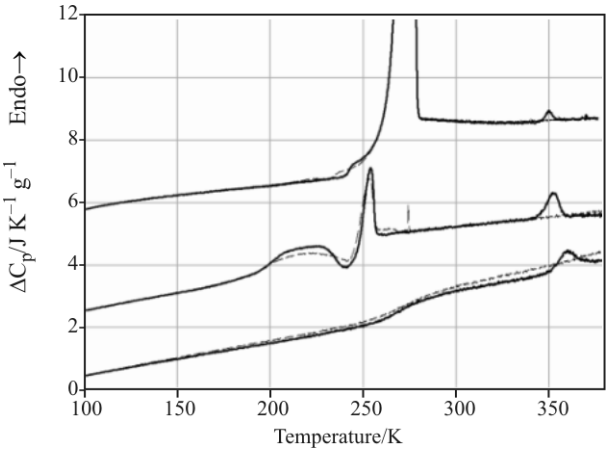

Fig. 2 Typical C_p *vs.* temperature curves for myoglobin–trehalose–water systems normalized to sample mass. From top to bottom: 270, 17, 4 water/ trehalose mole ratio. Solid lines: 1st scan; dashed lines: 2nd scan (after protein denaturation)

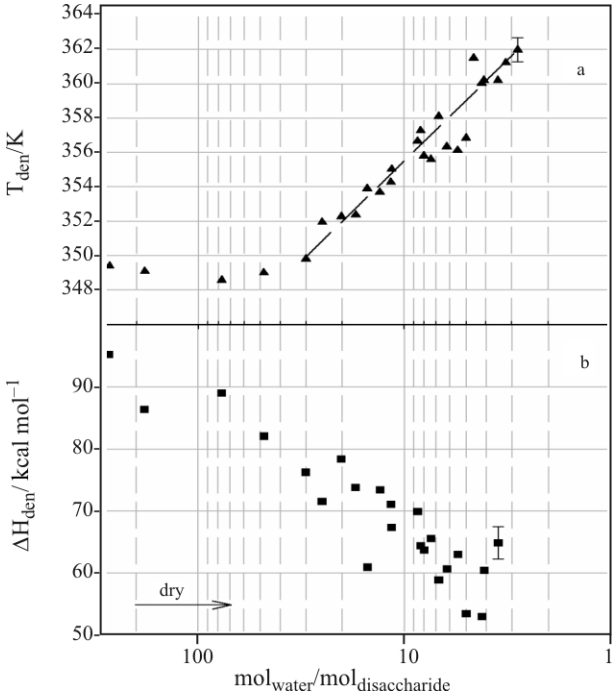

Fig. 3 a – Denaturation temperature and b – denaturation enthalpy of myoglobin as a function of hydration

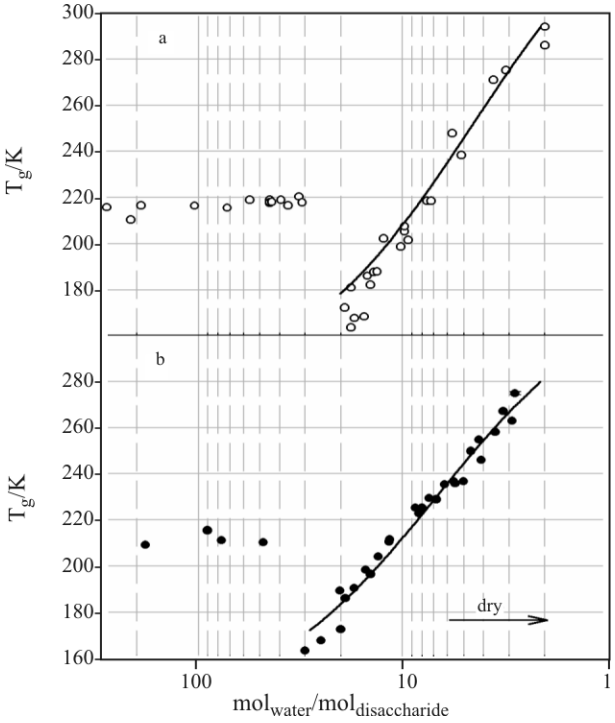

Fig. 4 Glass transition temperature of a – binary trehalose–water systems and b – of ternary Mb–trehalose–water systems as a function of hydration. Lines are fittings in terms of the Gordon–Taylor formula [34], performed by keeping T_g of water equal to 136 K [35]. Fitting parameters are in full agreement with literature [4–8]

glass transition, the exothermic/endothermic peaks due to water crystallization/melting and the endothermic peak due to myoglobin denaturation are clearly identified. Figures 3a and b show the myoglobin denaturation temperature and enthalpy, respectively, as a function of hydration, i.e. of the water/trehalose molar ratio, at constant protein concentration. Two main behaviours are distinguishable in Fig. 3a: at water/sugar mole ratio >30 (corresponding to ~1500 water/myoglobin mole ratio) T_{den} has an almost constant value ~349 K, which, by decreasing hydration, sizably increases up to 360 K for hydration levels ~3 water/sugar molar ratio. The T_{den} increase, evident in Fig. 3a, is not observed in the case of myoglobin in glycerol–water solutions [32]. A comparison between ΔH_{den} and T_{den} (Fig. 3) interestingly shows that, by decreasing hydration, T_{den} increases while ΔH_{den} decreases; this implies a larger entropy than enthalpy decrease and suggests that the effect of trehalose on myoglobin denaturation is an entropy driven process.

Figures 4a and b show the glass transition temperature of the binary water–trehalose systems and of the ternary myoglobin–trehalose–water systems, respectively, as a function of the water content. Data in Fig. 4a display the well-known T_g behaviour of aqueous binary systems. At high hydration levels the systems is inhomogeneous: a large part of the water component crystallizes, while only a small fraction of the system vitrifies at a constant T_g value, which corresponds to the concentration for the maximally freeze-concentrated solute matrix (C_g') [33]. Such behaviour is maintained in our binary system down to ~20 water/sugar molar ratio under our cooling conditions. At low hydration levels the system is homogeneous and vitrifies: the glass transition temperature depends on hydration and obeys the Gordon–Taylor expression [34] . As Fig. 4b shows, also the ternary system behaves as a binary one, in which the C_g' value is still ~10 water/sugar molar ratio (i.e. ~500 water/myoglobin molar ratio), while the region in which T_g keeps constant extends to ~30 water/sugar molar ratio.

Figure 5 shows the denaturation temperature of Mb *vs.* the temperature of the ternary system matrix glass transition: a linear correlation among these quantities is evident. This suggests a tight coupling among the protein stability and the dynamics of the surrounding matrix. Furthermore, Fig. 5 shows that a linear correlation also exists between the denaturation temperature of Mb and the glass transition temperature of trehalose–water binary system. The whole set of data presented in Figs 4 and 5 evidences that the presence of the protein does not sizably affect the glass transition of the trehalose–water systems. This behaviour suggests that, in protein–trehalose–water ternary mixtures at low hydration, the peculiar structure and hydrogen bonding capabilities of the trehalose fully govern the dynamics and the thermo-

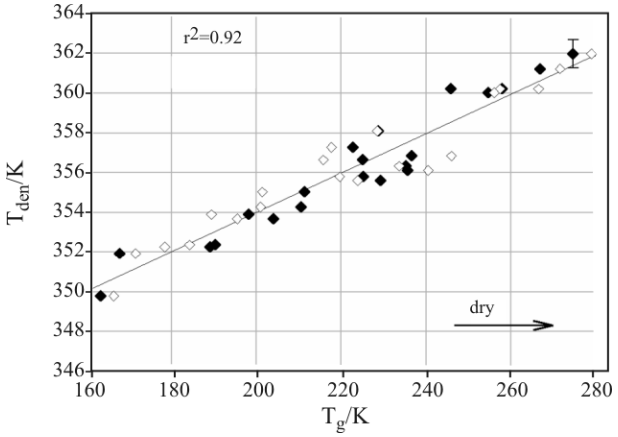

Fig. 5 Denaturation temperature of myoglobin as a function of glass transition temperature. ◆ – ternary Mb–trehalose–water systems; ◇ – binary water–trehalose systems. Solid line is a linear fitting

dynamics of the whole system. Furthermore, it agrees with the view that the trehalose peculiarity can be related to the high glass transition temperature of binary trehalose–water systems [4], although not rationalizing the role played by detailed molecular mechanisms. We are performing investigations on different saccharide systems aimed at understanding if and how peculiar differences, at molecular level, can affect the relationship between the protein denaturation and the glass transition of both the ternary and the binary systems, respectively.

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