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Water interplay in trehalose polymorphism

Attilio Cesàro *, Ornela De Giacomo, Fabiana Sussich

Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Via Giorgieri 1, 34127 Trieste, Italy

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Abstract

An investigation on the transformation paths of trehalose polymorphs and the interplay of water molecules is presented with the aim of underlining the role of trehalose structural and dynamic functions in the "protection" of biosystems and living organisms. To this end, physico-chemical studies have been carried out on water–trehalose interactions and on the structure and stability of the several forms, from the solution to the solid state (either amorphous or crystalline). In this paper the relevant results are critically presented and discussed with particular reference to most recent findings. The dehydration process performed under different confined conditions and controlled scan rates provides a dynamic phase diagram as a function of the time-scales of trehalose transformations and water effusion. A careful analysis of these data and of the stability of the glassy state in the presence of water evidences the reversibility of some trehalose transitions under the same conditions of natural processes of slow heating and controlled evaporation rate. This observation points at the hypothesis of the occurrence of these reversible paths at the basis of the bioprotection process.

In this paper, the relevant results from our laboratory and literature are critically presented and discussed in reference to the most recent findings.

Keywords: Dehydration; Trehalose; Polymorphism; Water; Bioprotection

1. Introduction

An overview of the most interesting aspects of trehalose bioprotective action is given, with the intent to provide an insight into the way water mobility and sugar structure can be related to each other. The authors' choice to focus the attention on the dehydration process and mechanism of the sugar trehalose is surely motivated by their own interest, but also amply justified by the role of this sugar in nature and by the relevance of the dehydration mechanism in the understanding of bioprotection. The dehydration process and the possible underlying mechanism have been recently studied and discussed in two articles in Nature Materials by developing a molecular picture of the organization and mobility of water in the amorphous and crystalline hydrate polymorphs (Cesàro, 2006; Kilburn et al., 2006). This inspired an artistic connection between the continuous dehydration and ensuing structural relaxation process with an Escher illustration conveying the sense of plasticity to such a relevant life process (see Fig. 1, in Cesàro, 2006).

Although trehalose is not yet used as a food preservative, its natural activity and its approval in food and pharmaceutical use make this molecule an extremely suitable mean for controlling water mobility and dry food stability. The historical perspective and the recent structural and thermodynamic features are then combined to develop a vision of a potentially general application of the preservation processes to all biosystems, from cells to foods, from drug delivery to nanobiotechnologies.

1.1. A bit of history, terminology and biosynthesis

The name trehalose derives from a desert manna, trehala, where the French chemist Berthelot found this sugar (Nwaka & Holzer, 1998), even though Wiggers had already discovered it in the rye ergot in 1832. It is widely present in

* Corresponding author. Tel.: +39 040 558 3684; fax: +39 040 558 3691.
E-mail address: cesaro@units.it (A. Cesàro).

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simple organisms as bacteria, rotifers, tardigrades, nematodes, but also in lichens, mushrooms and higher plants, reaching even 20% of their dry weight. Trehalose widespread presence in nature is associated with an unique important biological function.

In the biosphere many micro-organisms have developed a peculiar adaptation to environmental stresses by suspending their metabolism and becoming deprived of visible signs of life, and therefore isolated from harsh changes. When the original conditions are restored they are able to return to normal life and activity. There are several terms used in the literature to describe this state as viable lifelessness, suspended animation, viability and latent life but the very widely used terms are anabiosis and anhydrobiosis. The term anabiosis (or return to life) was introduced by Preyer (Keilin, 1959) for the phenomenon of resurrection of complete lifeless, but viable, organisms while the term anhydrobiosis was introduced by Giard in 1895. Another term nowadays recognized is cryptobiosis, latent life that was instead coined by Keilin, to indicate a state in which the metabolic activity is very hard to measure and the organism shows no signs of life. The general term hypobiosis summaries different states: anhydrobiosis due to dehydration, cryobiosis due to cooling, anoxybiosis due to lack of oxygen, osmobiosis due to high osmolytes concentration.

The discovery that very simple organisms can survive dehydration is dated back to 1702 (Keilin, 1959) thanks to Leeuwenhoek's observations written in a letter "on certain animalcules found in the sediments in gutters of the roof of houses". His first surprisingly observation was that the "animalcules", kept out of the water, contracted themselves into oval figures and that, after a short time of having poured some rain water, they began extending their body. He repeated the experiments several times, and noticed that even after having kept the "animalcules" in the dry state for several months, in few hours following rehydration, many of them returned to life. Leeuwenhoek's work was continued by other scientists on several types of organisms, like Doyer's extensive study on the effect of the drying temperature, or the contribution of some Italian scientists like Fontana, Roffredi and Spallanzani (Keilin, 1959) to reversible dehydration–rehydration understanding.

Life of tardigrades can be suspended for as long as 100 years. Their metabolism can be lowered to less than 0.01% and their water content can even fall to 1%. "Large" animals are not capable of withstanding a complete desiccation as some micro-organisms, but earthworms and leeches can loose very high proportions of their water, up to 93%, corresponding to about the 70% of their total body weight (Keilin, 1959). The time an organism can withstand dehydration changes form type to type and depends on the way desiccation is reached; the highest percentage of survival can be obtained if the evaporation is carried out very slowly, in 12 h (Caprioli & Ricci, 2001). An example of longevity is that of a plant seed of water lily or sacred lotus, *Nelumbo nucifera*: 85% of some seeds kept in the British Museum for 150 years were able to germinate. Furthermore, seeds of *Nelumbo nucifera* found in the basin of a dried lake in Southern Manchuria reached 100% of germination. The astonishment doesn't come from the percentage of germination but by the fact that they were about 1040 (±200) years old, based on the determination of the residual ^{14}C isotope. For the chronicle, on the basis of some geological consideration, the estimated age was indeed some 50000 years, but the scientist underlined that to estimate the maximum age, more experiments were needed on the seed shells and on the peat containing them. Besides this amazing case of longevity, there are several other examples. Some bacteria have been kept in the dry state for 30–45 years, and fungi were capable to produce active spores even after being kept dry in sealed tubes in vacuum for 35 years and in one case after 3 weeks having been spent in liquid air (−190 °C). Indeed, the effect of anhydrobiosis is different for different species: once returned to active life the time spent in the dry state has no effect on rotifers while life history of nematodes is affected (Ricci & Caprioli, 1998).

As far as trehalose biosynthesis is concerned, at least three pathways are known (Nwaka & Holzer, 1998; Singer & Lindquist, 1998). The most important one was described for *Saccharomyces Cerevisiae* and involves the enzymatic trehalose-phosphate synthase (Tps) complex. First, a glucose unit is transferred from uridine-diphopshate-glucose (UDP-glucose) to glucose-6-phosphate forming trehalose-6-phosphate and UDP, the reaction being catalyzed by Tps1p. Then, Tps2p phosphatase acts on trehalose-6-phosphate, removing the phosphate and yielding trehalose. At least two other enzymes are involved in this process: Tps3p and Tsl1p. Although their function is not completely clear, they have been proven to interact with Tps1p and Tps2p, and seem to contribute to the regulation of trehalose production.

2. Experimental section

Since in this paper several well-known methodological approaches are used to characterize the trehalose polymorphs and their inter-transformations, only a short guided reference to published work reporting the experimental techniques is given here.

Differential scanning microcalorimetry DSC, with emphasis of the confined condition control has been dealt by Sussich, Bortoluzzi, and Cesàro (2002), and in the references therein. The description of the time-resolved SWAXS experiments is provided in Sussich (2003). Molecular mechanics (MM) and molecular dynamics (MD) computer simulations have been carried out, following the general outline given by Liu, Schmidt, Teo, Karplus, and Brady (1997) and further described by Lefort, Bordat, Cesàro, and Descamps (2007a, 2007b); the latter reference describes also the experiments and the results of the solid state CPMAS NMR work on amorphous trehalose. Amorphous

trehalose–water system has been studied by Brillouin inelastic scattering in the UV range as described by Cesàro et al. (2007). All other results here quoted are given a proper reference to the original articles.

3. Trehalose physico-chemical properties

Trehalose (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside), also called *Mycose*, is a non-reducing disaccharide, dimer of glucose in which the two glucose units are linked in a α, α-1,1-glycosidic linkage (Fig. 1).

Of the three possible isomers, α, α-, α, β- and β, β-trehalose, the only naturally occurring form is the α, α isomer. From the chemical viewpoint both the presence of symmetrically linked glucosidic residues and the absence of reducing groups make this molecule very peculiar. Among naturally abundant sugars, only sucrose presents the same characteristic of chemical stability in solution because of the absence of anomeric forms.

The physico-chemical properties of trehalose are often described as "anomalous" in the attempt to justify the important biological role of trehalose, and are especially described as "peculiar" near the saturation or the glass transition lines. In the following section, a critical summary of trehalose properties is reported, with the main purpose of rationalization of the relevant features and disclosure of some incorrect literature data. The latter have at least partially fed the trehalose myth, in terms of uniqueness of its properties. The presentation moves from the crystalline states and the glassy form, including their phase transformation to the solution state. Under several aspects, trehalose properties may appear not very different from all other similar sugars, like sucrose and maltose. Nonetheless, some small differences may contribute to an overall more peculiar dynamic picture.

Fig. 1. The sketch of the chemical structure of α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside showing the particular glycosidic linkage connecting the two reducing units.

3.1. Crystal structures and transitions

Trehalose has been known for a long time to possess two different crystalline states, one of them involving two water molecules per disaccharidic unit (trehalose dihydrate, TRE-h). The crystal structure (Brown et al., 1972; Taga, Senma, & Osaki, 1972) contains four sugar units $C_{12}H_{22}O_{11} \cdot 2H_2O$ within an orthorhombic cell ($P2_12_12_1$). Water and trehalose molecules are held together in the crystal (Fig. 2) by a complex system of 12 hydrogen bonds where every hydroxyl group of the trehalose molecule is both donor and acceptor in the hydrogen bond network. However, in the crystal there are no intramolecular hydrogen bonds such as in sucrose or cellobiose. Furthermore, the two water molecules are not equivalent, since they form two different types of hydrogen bonding. One water molecule is an acceptor of two bonds and its oxygen is tetrahedrally coordinated, while in the other water molecule the oxygen is pyramidally coordinated and it is acceptor only for one bond. This different type of bonding created by the water molecules produce a helical arrangement.

Trehalose can also crystallize in an anhydrous form, TRE-β, (Jeffrey & Nanni, 1985) with a monoclinic ($P2_1$) crystal structure in which all the hydroxyl groups are involved in the network of hydrogen bonds. In the anhydrous crystals both the ring-oxygen atoms are involved in the hydrogen bond while the glycosidic oxygen atom is not.

Given the presence of (at least) two different crystalline forms, transitions from one form to the other are therefore possible (see Table 1 for the thermodynamic parameters). The first report on trehalose transitions was that of Reisener, Goldschmid, Ledingham, and Perlin (1962). A sintering and birefringence loss of TRE-h was observed at 100 ± 3 °C, while a melting occurred at 135 °C. Melting of the anhydrous form TRE-β (prepared at $T > 130$ °C) occurred at 216–218 °C. The same authors also reported the preparation of another crystalline form, called here TRE-α. Only after some time, the TRE-α form has

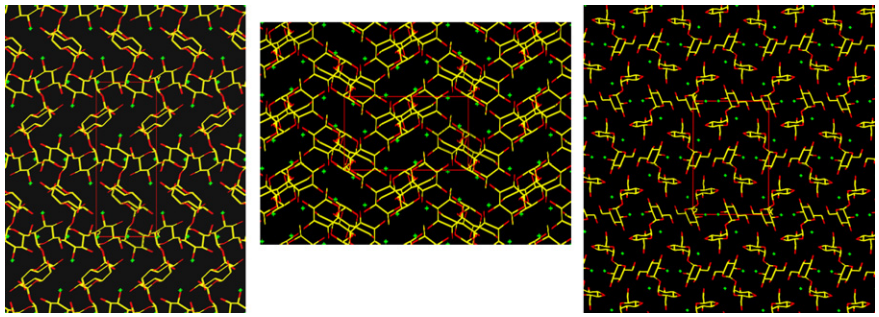

Fig. 2. Tridimensional view of dihydrate trehalose (TRE-h) along axis (left), (center) and (right); only the sugar ring (yellow color) with oxygen (red) and the oxygen of the water molecules (green) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of transition temperatures and thermodynamic data for trehalose polymorphs[a]

Transition	T_{tr}/K (/°C)[b]	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}
Glass transition: $T_g \rightarrow T_{am}$	393 (120)	(0.48 J K^{-1} g^{-1})[d]	
Dehydration of T_h : $T_h \rightarrow$ anhydrous	373 (100)	113.5 (full)[c]	304[c]
Dehydration of T_h : $T_h \rightarrow T_\gamma$	373 (100)	52[c]	139[c]
Dehydration of T_h : $T_h \rightarrow T_\alpha$	<373 (<100)	113[c]	300[c]
Melting of T_α : $T_\alpha \rightarrow T_{am}$	399 (126)	*ca.* 10[a,d]	14
Transition of T_γ : $T_\gamma \rightarrow T_\beta$	393 (120)	51.3	130
Melting of T_β : $T_\beta \rightarrow T_L$	478 (205)	51.3	107

[a] Taken from Sussich and Cesàro (2000).
[b] Onset temperature.
[c] The value includes the two water molecules undergoing vaporization: TRE-h (crys) → TRE-*i*(solid) + 2H_2O(g).
[d] Taken from Willart et al. (2002).

attracted the interest of many researchers because of its low melting point and its hidden crystalline structure, still unknown (Sussich, Urbani, Princivalle, & Cesàro, 1998; Sussich, Princivalle, & Cesàro, 1999; Sussich & Cesàro, 2000; Sussich, Skopec, Brady, & Cesàro, 2001; Willart et al., 2002; Willart et al., 2006). These literature findings have been re-proposed by Sussich et al. (1998, 1999) and quoted in a survey mostly devoted to the supplemented phase diagram (Chen, Fowler, & Toner, 2000).

Shafizadeh and Susott (1973) reported DSC thermograms showing two sharp endotherms at 100 °C and 215 °C, for the melting of TRE-h and TRE-β, respectively. However, their thermograms (at 5–15 K/min) showed more endothermic peaks whose complexity was attributed to the presence of amorphous glass that undergoes liquefaction.

In his seminal review on melting and glass transitions of low molecular weight carbohydrates, Roos (1993) reported the known data on melting temperatures of trehalose. The onset and peak temperatures of melting of the dihydrate crystal are 91 °C and 97 °C, respectively, while the onset temperature of 203 °C, given for the final melting of the anhydrous TRE-β, was taken from Slade and Levine (1991). A value of 100 °C was reported in a subsequent paper (Ding et al., 1996) as a T_g value (indeed!), but with the relevant information that, when rapidly cooled to −100 °C, solutions containing from 70% to 90% of sugar were unable to show glass transition upon re-heating. Rapid crystallization of hydrate form was inferred. In a previous paper, Green and Angell (1989) reported that the melting transition of trehalose dihydrate occurred in the range 90–100 °C (Fig. 1 of Green & Angell, 1989). Subsequently (Ding et al., 1996), a small endothermic peak at 125 °C was observed for the first scan after dehydration and attributed to an anomalous glass transition (the doubt over a possible metastable state was also mentioned). In our laboratory the different polymorphs have been prepared and their transformations studied in detail (Sussich et al., 1998). In addition to the dehydration of TRE-h (100–110 °C) and melting of TRE-β (215 °C), the melting of two forms, TRE-α (126 °C) and TRE-γ (118–122 °C) have also been reported (Fig. 3).

The latter was then characterized as a mixture of two crystalline forms, and probably made up of TRE-h crystals encapsulated in layers of TRE-β (Sussich et al., 1999). In Fig. 3 are depicted the thermograms of the different

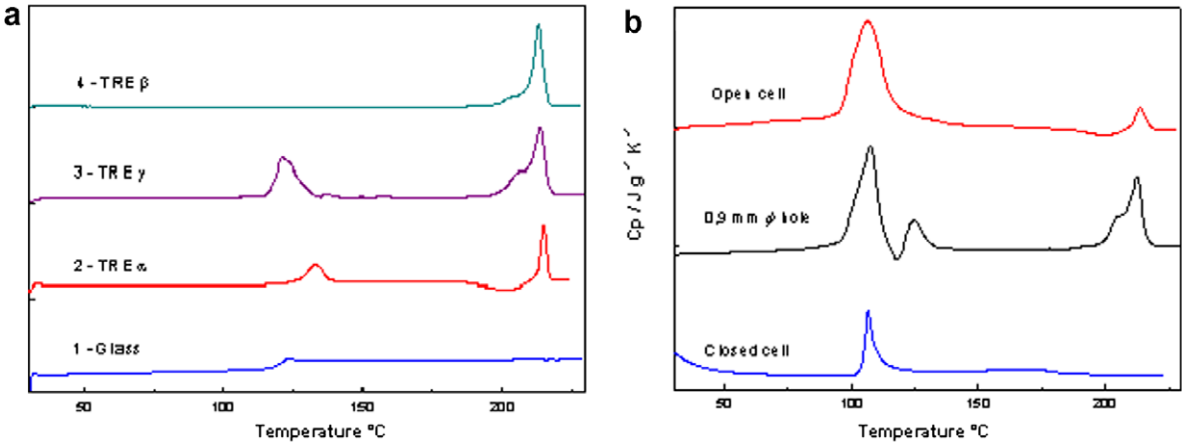

Fig. 3. Thermal behavior of trehalose polymorphs at scan rate 20 K min^{-1}. The thermograms in (a) show from bottom to top: (1) the glass transition, (2) the melting of TRE-α, followed by cold crystallization to TRE-β and its melting, (3) the dehydration of TRE-γ with solid-transition to TRE-β and its melting, (a) the melting of pure TRE-β. The thermograms in (b) show the dependence of the dehydration process of TRE-h under different regime of water effusion (from bottom to top: closed, pin-hole and open cells, respectively).

polymorphs, recorded at a scan rate of 20 K min^{-1}, to show the different thermal behavior of the polymorphs that have been extensively characterized by thermal analysis. The latter was then characterized as a mixture of two crystalline forms, and probably made up of TRE-h crystals encapsulated in layers of TRE-β (Sussich et al., 1999).

This type of studies has been deeply pursued in our laboratory and further results on phase properties and transformations are reported and discussed (Sussich, 2003). In conclusion, transition temperatures, transition enthalpies and non-equilibrium cold crystallization phenomena have well-defined values and contribute to the fingerprinting of trehalose polymorphs (Table 1).

It has been assessed (Sussich et al., 2002) that TRE-h behavior strongly depends on the heating rate but also on the time water remains in contact with trehalose crystals. The residence time is modulated, among other parameters, by the hypothetical size of the cell pores. By means of calorimetric investigations, the easiness of water molecules to leave the crystal environment has been correlated to the diameter of the hole on the calorimetric cell caps. Scan rate determines the vapor effusion and indirectly, water residence time upon heating scans (water plasticization). Modulation of the two streaming variables (temperature scanning and water flowing) is the key action for the production of a given trehalose form. This new type of analysis led to the construction of a dynamic diagram, reported in Fig. 4. Although not at equilibrium, it is referred to as a "dynamic phase diagram", because it identifies a particular polymorph in a domain of temperature, scan rate and water effusion. The diagram shows not only the "static" temperature/transformation coexistence lines but also their dependence on the time required for the transformations and the changes occurring as a consequence of the rate of vapor flowing out of the cell.

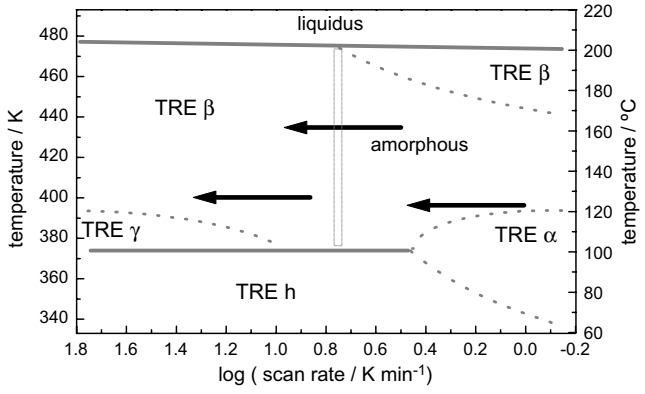

Fig. 4. Schematic diagram of thermal transitions of trehalose dihydrate (TRE-h) at different scan rates (note the inverse direction of *x*-axis to represent the time elapsed). Main transitions (dehydration and melting of TRE-β) are reported as full lines and are mostly independent of scan rates. For each scan rate, the possible formation of other polymorphs is shown in the regions delimited by dotted lines. Arrows indicate the effect of increasing the pore size of the calorimetric cell (see in Fig. 3b), i.e. decreasing the residence time of water vapor.

3.2. The glassy state

On the basis of the behavior of concentrated solutions of sugars it has been suggested that most of these systems easily form glasses when dried under suitable conditions at low temperatures. Therefore, the intuitive deduction is that the glassy state of sugars is involved in anhydrobiosis (Green & Angell, 1989; Crowe, Hoekstra, & Crowe, 1996a; Franks, 1990; Sun & Davidson, 1998). However, earlier data on the glass transition temperatures of trehalose were not always accurate nor agree each other (Shafizadeh & Susott, 1973; Green & Angell, 1989; Slade & Levine, 1991; Crowe, Reid, & Crowe, 1996b; Ding et al., 1996; Roos, 1993; Saleki-Gerhardt & Zografi, 1994; Sussich et al., 1998). Even more important is the effect of water plasticization on the glassy/amorphous carbohydrate properties that have been repeatedly reported and reviewed in the literature.

Only recently, among all available data, has a reasonable value of about 120 °C been accepted for the glass transition temperature (Chen et al., 2000; Sussich & Cesàro, 2000). This value of T_g is comparable with that of a tetrasaccharide (e.g. maltotetraose) and it is the highest in the disaccharide series, whose T_g's range mostly between 65 and 100 °C. However, an aleatory glass-like transition endotherm around 75 °C has been often observed in the preparation of anhydrous trehalose, although it is not clear whether this glassy state may contain a small amount of stoichiometric water. In other words, it is assumed that under some experimental conditions a segregated mixture of plasticized trehalose is formed. The bulk of the published data for trehalose/water system concerns the dependence of the glass transition temperature on composition. In the most recent review of such data (Chen et al., 2000), a fit of all experimental data provided the value of $k = 5.2$ for the parameter of the Gordon–Taylor equation (Gordon & Taylor, 1952). However, the same authors, although noting that "there was only poor agreement on the glass transition temperature of trehalose (variously reported between 73 and 115°C)" did not mentioned the higher values of 120 °C and 133 °C, given for the onset and mid-point transition temperature, respectively, by Shafizadeh and Susott (1973) and Sussich et al. (1998).

Nowadays, "state diagrams" report not only the thermodynamic equilibrium lines between the different phases (Lammert, Schmidt, & Day, 1998; Mehl, 1997; Miller, de Pablo, & Corti, 1997; Nicolajsen & Hvidt, 1994; Wang & Haymet, 1998), but also the "non-equilibrium" lines that can be achieved in the thermodynamic experimental timescale (usually of the order of minutes). However, in view of the different methodologies and the different time-scales used, one should more correctly express these lines at a definite value of the Deborah number (*De*). This parameter gives the ratio between the relaxation time of the molecular system undergoing the transformation and the observation time of the phenomenon (Reiner, 1964). Relevant to this transformation time is the observation that moistened

glassy trehalose appears unstable and that the molecular mobility in the plasticized glass may allow crystalline dihydrate trehalose to be formed (Aldous, Auffret, & Franks, 1995).

The greater molecular mobility of trehalose glass with respect to other sugars, such as lactose and sucrose, has been inferred in a recent study by Lefort et al. (2007a, 2007b), on the basis of solid state NMR and computational investigation on sugar glasses. The larger internal mobility of trehalose is however accompanied by the ability of trehalose to form larger clusters than sucrose (Lerbret, Bordat, Affouard, Descamps, & Migliardo, 2005). Subsequently, on the basis of the enthalpy relaxation studies of the three sugars, lactose, sucrose and trehalose, during isothermal aging, it has been shown that the size of the cooperative regions in the temperature range between 298 and 365 K is much larger for trehalose than for sucrose and lactose (Haque, Kawai, & Suzuki, 2006). As a further, still preliminary, result of our laboratory, some evidence for the existence of two amorphous phases in trehalose glass has been achieved by studying the structural relaxation of glasses prepared with different protocols (Pastrello, 2006).

The molecular mobility of "amorphous" trehalose and the properties of sugar–water systems at the freezing point of water (ice formation) raise another concept of great attention, in particular in food technology. In principle, upon formation of ice, dilute solutions concentrate to reach the critical lower T'_g temperature at the maximum concentration of solute C'_g (for trehalose/water $T'_g = -30\,^{\circ}C$ and $C'_g = 0.2\%$ (w/w), respectively). The T'_g–C'_g point is indeed a sort of non-equilibrium triple point among solid ice, liquid phase and plasticized glass at equilibrium. Large interest and debate are found in the literature on the determination of this point and on its practical importance in the aqueous sucrose system, just to quote the most relevant technological sugar. However, the expectation that dilute solution simply concentrates upon ice formation always excludes the possibility of a liquid–liquid phase separation or any other non-miscibility process (solubility limit). Contrary to sucrose – see the most recent data collection and modelling in Starzak and Mathlouthi, 2006, trehalose appears not only less soluble, but also offers a greater number of crystalline polymorphs, one of which, TRE-h, is much less soluble and therefore the solubility region – that is the liquid homogeneous phase – is strongly restricted. The temperature–composition phase diagram, such as that reported in Fig. 5, artificially enlarges the thermodynamic stability of the solution simply because of the use of the weight fraction scale, instead of the molar fraction scale. Independently of this consideration, it is clear enough that upon cooling (slowly) trehalose solutions, less concentrated than say 30% of trehalose, the first event is the formation and the separation of ice. Upon increasing concentration of trehalose the solubility line of TRE-h is encountered and, therefore, dihydrate trehalose TRE-h must be formed and phase separated in the eutectic composition with ice.

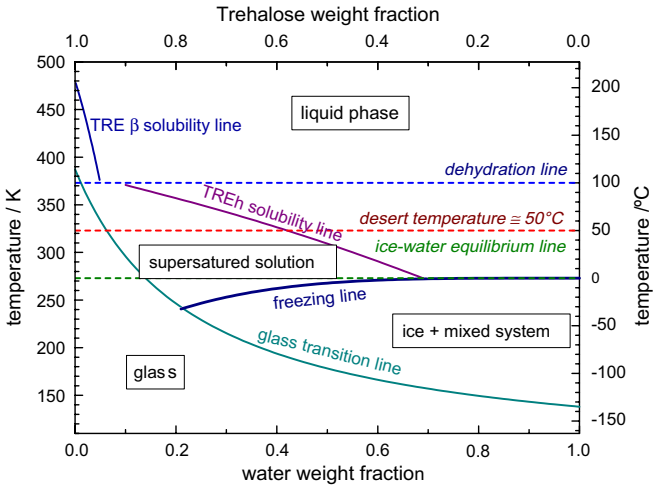

Fig. 5. The water–trehalose temperature–composition phase diagram. The composition is expressed as weight fractions of water (bottom axis) and of TRE (top axis). The transition lines define the solubility of the dihydrate, TRE-h, and anhydrous, TRE-β, crystals and the stability of the glassy state, in addition to that of water freezing from the solution. For visual purposes, the relevant temperatures of 0, 50 and 100 °C are marked.

The possibility that no crystalline phase separation occurs must also be taken into account, whenever the cooling rate and/or concentration rate is an effective damping to the equilibrium processes.

3.3. Trehalose transformations as seen by structural methods

Inter-conversion between trehalose polymorphs has been widely investigated by means of several thermodynamic and structural methods. In particular, dehydration of crystalline trehalose dihydrate TRE-h has been widely studied with both experimental and theoretical means (for a summary see refs Affouard et al., 2005; Kilburn et al., 2006; Sussich et al., 2002). Among the first observations, thermogravimetric analysis and DSC have been the most direct, albeit phenomenological, methods to detect the dehydration (see for example data reported in paragraph 3.1). Comparatively, little structural information seems to have been achieved from the few studies based on IR and XRD as far as the dehydration mechanism is concerned.

Although the characterization of the crystal structure of polymorph TRE-α has not been fulfilled, structural studies based on X-ray diffraction and scattering (powder XRD, SWAXS) and on spectroscopic methods (FTIR) give valuable information on the structural transitions. Moreover, if the structures of the different polymorphs are known, it is possible to assign unambiguously a pattern to any single species and follow their evolutions as time, temperature or humidity change.

Simultaneous DSC – SAXS/SWAXS experiments have been performed on dihydrate trehalose in the range 30–230 °C (Sussich, 2003). The solid–solid transition from TRE-h to the β-form was observed with an abrupt change in the diffraction pattern over a temperature around

100 °C. This transition is seen both with scanning rates of 10 K/min and 1 K/min, meaning that TRE-β is formed independently of the heating rates in sealed capillaries. In another experiment, where trehalose dihydrate crystals were placed in quartz capillaries under nitrogen flux, with a scan rate of 10 k/min, the integrated intensities of the main reflections of the polymorphs surprisingly revealed the presence of TRE-α, in coexistence with the dihydrate, already at 60 °C (Sussich, 2003). Parallel to the melting of the dihydrate into TRE-α, another phase (amorphous) appears in inferior quantities.

Simultaneous DSC – XRD and DSC – SAWXS experiments (Nagase, Endo, Ueda, & Nagai, 2003; Sussich, 2003) show the appearance of two peaks at diffraction angles of 16.1° and 17.9°, corresponding to the dehydration of the dihydrate into TRE-α (the latter authors refer to it as κ-form). The peaks of the TRE-h are lost at about 100 °C, in coincidence with the DSC endothermic peak, ascribed to the melting of the TRE-h. The polymorph TRE-α disappears above 130 °C as seen by DSC and a halo pattern in XRD at $T > 130$ °C reveals its transformation into an amorphous phase. Thereafter, the amorphous lately evolves in TRE-β by cold crystallization at $T < 190$ °C. Eventually, the endothermic melting peak of TRE-β at 210 °C is paralleled by a halo pattern in XRD at $T > 210$ °C. All these results are in general agreement with FTIR data of Akao, Okubo, Asakawa, Inoue, and Sakurai (2001), who studied the transitions of TRE-h in the temperature range from 40 to 160 °C, by monitoring the stretching bands of the glycosidic linkage around 1000 and 950 cm^{-1}. Furthermore, given the water bending at 1600–1700 cm^{-1} and OH stretching at 3500 cm^{-1}, the same authors have been able to recognize that dehydration occurs at 80 °C and that the sample undergoes some significant structural transition at 80 °C and 130 °C (respectively, formation and melting of TRE-α).

It is clear enough, however, that variations in the transition temperatures here reported must refer to the specific experimental set-up chosen by the authors, in view of the dynamic phase diagram reported in Fig. 4. Once more, this diagram provides the rationale for the understanding of the parameters controlling the several transitions between polymorphs and will be the basis for a description of a possible mechanism in the next paragraph.

3.4. Solution properties

An overview of the solution properties of trehalose is also needed in view of the well-known stabilizing activity of sugars toward preserving native conformational structure of proteins. In the last decades, it has been popular to interpret the variations of the solvent properties in terms of "water-structuring" and "water-destructuring" effects. The past meaning of these terms is now abandoned, as dynamic pictures of the structure of water are being developed. Still, the ability of sugars to protect biological structures both in semi-dilute and concentrated solution cannot be disregarded. Therefore, aqueous solution properties of trehalose have been deeply studied experimentally and modeled by computer simulation; for some references see the original work of De Pablo and coworkers, Magazù and coworkers, and the other references quoted by Sussich et al. (2001).

The attention of many workers is addressed to accurately determine the hydration number of trehalose in order to disclose peculiarity in comparison with other similar molecules, namely sucrose and maltose. A recent analysis of the hydration properties of small sugars has been based on the non-ideality of the equilibrium hydrated species (Ben Gaÿda, Dussap, & Gros, 2006). Ultimately, the accurate determination of the number and the strength of hydrogen bonds between solute and solvent will provide the answer to the question if trehalose involvement in bioprotection resides in its solution properties. The more general problem is the correct definition of water molecular clustering around a trehalose molecule, which ultimately affects long-range mobility and local dynamics. Solvation shells are characterized at molecular level by a large fluctuation, which provides different statistical averages in the dynamic (e.g., by dielectric relaxation) and static measurements (e.g., by density).

There have been also quite a few computational studies on trehalose and its hydration (Bordat, Lerbret, Demaret, Affouard, & Descamps, 2004; Donnamaria, Howard, & Grigera, 1994; Engelsen & Perez, 2000; French, Johnson, Kelterer, Dowd, & Cramer, 2002; Liu et al., 1997; Sakurai, Murata, Inoue, Hino, & Kobayashi, 1997). These studies primarily focused on questions relative to the conformation of trehalose in solution, its degree of hydration, and the extent to which this solute perturbs solvent water. None of these simulations found any solution behavior that was strikingly different from that of other disaccharides, such as an anomalously large hydrated radius. However, characteristics were seen which might explain why trehalose is the outliner in values of properties like glass transition temperature (Engelsen & Perez, 2000). Based on the analysis of the distribution of intermolecular distances (Voronoi volumes), the most recent investigation (Bordat et al., 2004) assigns to trehalose a slightly stronger solvation. This fact has correspondence in other MD simulations (Engelsen & Perez, 2000; Liu et al., 1997) that have concordantly found one water molecule very often resident in a position that corresponds to that of one of the two water molecules in the crystalline TRE-h.

Therefore, the apparently strong interaction of water with trehalose would easily justify the appearance of a crystalline dihydrate TRE-h form at relatively moderate concentration. Indeed, it is a thermodynamic rule that solubility of crystalline hydrates is smaller than that of anhydrous forms (see the phase diagram in Fig. 5). However, the thermodynamic significance of solubility lines has not been discussed in detail for correlation with other properties that are also known, and, in particular, the thermodynamic data of trehalose have never been analyzed by taking into account the non-ideality of the saturated solution of non-electrolyte mixtures. Thus,

there is still some incongruence, for instance in the solubility properties, and it has still to be ascertained if this is due only to kinetic effects or to the presence of a metastable monohydrate form.

Recently, Brilloin inelastic scattering experiments have been carried out by using synchrotron radiation in the UV wavelength range (Cesàro et al., 2007). The most relevant phenomenological point is that the trehalose–water system investigated is an undercooled liquid from the thermodynamic time-scale, while the glassy character has already been shown at the frequency of this study (cf. Deborah number). The dependence of α-transition and sub-T_g transition temperatures with the measurement frequency is well known in the field of polymers, by studies with calorimetric, dynamico-mechanical and dielectric methods. It opens, however, a new scenario on the time-scale of the dynamic transformations of trehalose.

4. An insight to trehalose dehydration mechanism and the bioprotection hypothesis

Despite the enormous amount of work and experiments carried out in the last three centuries, the mechanism by which trehalose acts appears still unsolved. There are, although, several hypotheses. The several explanations proposed in the past are only briefly reported below, while details and other related aspects are found in comprehensive reviews.

The chemical stabilization, proposed in the 1970s, has been revisited to explain the protection exhibited by complex molecular structures formed by chemical reactions between sugars and biomolecules at high temperatures. The formation of protective films is due to chemical reactions of the Maillard and Amadori type and, therefore, cannot be relevant for the in vivo, reversible action of trehalose and similar sugars since trehalose a is stable non-reducing carbohydrate and is hydrolyzed only at high temperatures, or in acidic conditions. The absence of free reducing anomeric carbon in trehalose and its non-reversible action are the main reason for ruling out a mechanism which could involve these reactions under physiological conditions (Shafizadeh, McGinnis, Susott, & Tatton, 1971).

In the water replacement hypothesis, the emphasis is put on the dependence of the three-dimensional structure of biological macromolecules on the stabilizing effect of a water molecular layer which effectively interacts with surface residues via hydrogen bonding and electrostatic–polar interactions. It is accepted that the amount of water involved in the stabilization of globular proteins is of the order of 25–75% w/w (water/protein). On this basis, in their initial proposal, Crowe, Hoekstra, and Crowe (1992), and recently other authors suggested that sugar molecules were able to form large three-dimensional networks of hydrogen bonds both within the sugar molecules and with peptide groups, therefore protecting the conformational stability of biomolecular structures. Such interactions were found to stabilize the aqueous proteins with sucrose. This proposal surely arose from the extensive work on aqueous solutions of model biomolecules which suggested the words "structuring" and "destructuring" for the effects of some solutes on the so-called "water structure". Water structure was depicted in the 1960s as an effect of dynamically organized clusters of water molecules ("flickering clusters"), originated by the presence of a fraction of hydrogen bonds in the liquid water. A two-state model was widely used to explain the hydrophobic effects as well as the perturbations that other non-ionic solutes were able to induce in the properties of the solvent, toward either an increase or a decrease in water structural organization. However, later studies of molecular thermodynamics and of relaxation spectroscopies on these systems showed that "water structure" must be viewed in a more dynamic way and that sugars protect the native state of protein by destabilizing their unfolded conformation. Furthermore, recent investigations by means of molecular dynamics on carbohydrates in solution have modified the original picture of the static hydration pattern of sugar molecules. Therefore, it is highly improbable that the hydrogen bond fingerprint of sugars could be so specific and in register, albeit dynamically, with such a large number of biomolecular structures.

Concerning the third hypothesis, it is well known that sugars, once melted, can easily be cooled without undergoing crystallization. The amorphous to glassy state transition for most sugars occurs about 100 K below their melting point and many cryoprotectant sugars can be prepared in a glassy state at ambient temperature. It was therefore straightforward to suggest that this ability to form a glassy state is relevant for the protective action of sugars during severe dehydration. It is thus generally accepted that, upon decreasing temperature or increasing concentration, the viscosity of the aqueous solution increases above the reasonable limit for crystallization kinetics to be manifested. This means that diffusional processes slow down to the point that the amorphous (crystallizable) system can be further cooled or concentrated, undergoing a glass transition. Among all hypotheses, the formation of glassy layers of sugar molecules has received the wide credits from the physical point of view. For this aspect, the high efficiency of trehalose has been ascribed to the high value of the glass transition temperature, an explanation that has been rejected however by other researchers in view of the minor effect of oligomers or dextrans with higher T_g values.

On the basis of results on fusion and aggregation of vesicles, Crowe et al. (1996a) arrived at the conclusion that vitrification is not the only mechanism by which trehalose and other sugars impart their bioprotection, but that also direct interaction has to be invoked. Therefore, it was suggested that three combined factors (Tm depression, direct interaction and glassy state formation) are involved in membrane stabilization. In conclusion, although an enormous amount of data has been collected over the years, there are still

open questions about the protection mechanism. This point is explicit in two recent papers where Tunnacliffe, de Castro, and Manzanera (2001) Crowe, Oliver, and Tablin (2002) wonder if there is really a single protection mechanism and whether this mechanism will ever be fully understood.

We return now to the trehalose dehydration process and to the speculation early made that reversible transformation paths among trehalose polymorphs may be a clue for the clarification of its bioprotective action (Sussich et al., 1998, 1999, 2001). In general, the transformations of hydrated polymorphs of sugars are tuned by the rates of temperature change and water removal. Trehalose, with its three crystalline polymorphs (one dihydrate form, TRE-h, and two crystalline anhydrous forms, TRE-α and TRE-β), and the fact that its glassy state has the highest transition temperature of all disaccharides, is unexpectedly different from the homologous disaccharides maltose and sucrose, with lower glass-transition temperatures and less polymorphs.

A further relevant property of trehalose is that the addition of water to an amorphous phase generally facilitates an increase in mobility and therefore a drop in the glass-transition temperature. However, it has been postulated that the reason the "apparent" glass transition of amorphous trehalose is still high, even in the presence of residual (or added) water, is that TRE-h dihydrate crystallites are locally formed within the amorphous matrix. Thus, the ability of amorphous trehalose to capture moisture into the cages of TRE-h crystallites, coupled with the reversible transition between the dihydrate form TRE-h and the anhydrous form TRE-α achieved by extraction of water from the TRE-h form, provides the perfect platform for water immobilization during dehydration. Measurements of the free volume of trehalose amorphous phase show that water inclusion simply increases the average intermolecular hole size. In the crystalline dihydrate, TRE-h, however, the water is confined as a one-dimensional fluid in channels of fixed diameter. These channels allow diffusion of water in and out of the crystallites, and can therefore act as both a sink and source of water in low-moisture systems. The energetics of water escaping process has been modeled by Molecular Dynamics simulation and experimentally determined by Differential Scanning Microcalorimetry (Sussich, 2003). These results show the different energetics of thermodynamic and kinetic nature given to the two different types of water molecules in the crystalline TRE-h, as well as the origin of the reversible paths in the dehydration process. Thus, trehalose polymorphism and the formation of a water-immobilizing crystalline-glassy nanocomposite phase of TRE-h crystallites in the amorphous matrix seem to provide a reasonable basis for the survival of biological functions by dehydration.

5. Conclusions

In this work a summary of trehalose physico-chemical properties is given, with special emphasis on the amorphous and crystalline phases. The presentation is organized as to provide the following conclusions:

(1) The several trehalose polymorphs are identified by their structure and thermodynamic properties, but the lack of knowledge of the anhydrous *TRE-α crystalline* structure is striking.
(2) The influence of temperature on the transformation among different polymorphs is studied mainly by microcalorimetry, but also by structural methods, both in isothermal and scanning conditions.
(3) The effect of water on the stability of the several phases and the role of the glassy state in the compositional phase diagram are discussed also in relation with trehalose solution properties.
(4) Finally, the thermodynamics of the dehydration process of dihydrate trehalose crystals and the new recent findings on the structural changes at a nanoscale level are merged. This analysis reinforces the original proposal of reversibility as the basis for the bioprotection process (Sussich et al., 1998; Kilburn et al., 2006; Cesàro, 2006).

The physico-chemical properties and the related conclusions on the versatile transformations of trehalose have, therefore, a fundamental role in view of the in vivo function of bioprotectants. Further attention should be given to the unexplored potential of trehalose as "specialty" and "commodity".

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