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Perspective

Reversible dehydration of trehalose and anhydrobiosis: from solution state to an exotic crystal?

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Abstract

Physico-chemical properties of the trehalose–water system are reviewed with special reference to the transformations that may shed light on the mechanism of trehalose bio-protection. Critical analysis of solution thermodynamics is made in order to scrutinize trehalose properties often called 'anomalous' and to check the consistency of literature results. Discussion on the conversion between the solid state polymorphic forms is given, with a special emphasis of the transformations involving the newly identified anhydrous crystalline form of α,α-trehalose, TRE_α. This exotic crystal is almost 'isomorphous' with the dihydrate crystal structure, and possesses the unique feature of reversibly absorbing water to produce the dihydrate, without changing the main structural features. The reversible process could play a functional role in the well-known ability of this sugar to protect biological structures from damage during desiccation. The final aim of the paper is to add some new insights into and to reconcile previous hypotheses for the peculiar 'in vivo' action of trehalose.

Keywords: Trehalose; Thermodynamics; Polymorphism; Hydration–dehydration; Anhydrobiosis

1. Introduction

Nature shows many examples of strategies for long term survival of organisms. Among those of most interest is the protection of living organisms against low-humidity in extreme temperature conditions, far from the ambient. Definition of these anhydrobiotic processes is not easy nor has the nature of the mechanism(s) been clarified: it is therefore not semantic to refer to the phenomena as 'cryptobiosis', to underline the cryptic ('unknown') way living systems are preserved. The phenomenological observation is that anhydrobiotic organisms can be reversibly dried and then re-hydrated without cumulating effects of functional stresses.[1] This suggests that the same process could be effectively used in preservation technologies, which are very important for high-value biological products.

Sugars are the most well known chemicals that Nature and man use as stabilizers for complex biostructures and as food preservatives. Globular proteins invariantly show higher melting (denaturation) temperatures in the presence of sugars (in dilute solution), and

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concentrated sugar systems confer textural consistency that prevents microbial attack in foods. It is not clear, however, whether these two different types of stabilization have a common physico-chemical origin, and in particular whether water–sugar interactions are the only basis of both phenomena. The reason to relate these two stabilization processes is that in Nature, production of protective sugars is triggered by the detection of low-humidity and/or high/low-temperature by the organism; under these conditions, sugar concentration increases with time, passing from very dilute solution to solid-like highly concentrated bio-mixtures. During this slow dehydration process, even if the water content falls below the critical value of 20–30%, cells do not degrade and, upon subsequent re-hydration, biomolecules maintain the native conformation necessary for cellular functions.

Among all the sugars, trehalose has received the greatest attention, both because of its wide role in Nature and its potential use as a highly efficient natural preservative.[2,3] This non-reducing disaccharide of α-D-glucose is commonly found in mushrooms and drought-adapted organisms.[4] In addition to being present in these organisms, the disaccharide accumulates in spores, yeasts, and in the cells of *Rhizobium leguminosarum* 'as osmoprotectant parallel to the increasing osmotic pressure of the medium'.[5] Experiments have revealed that all these organisms are able to induce the production of trehalose as they desiccate, and the dried organism is able to survive in a dormant state and then to 'resuscitate' when environmental humidity permeates the cell, restoring the original conditions; this state can also be induced in non-adapted cells through the addition of exogenous trehalose. Trehalose has been pictured as acting like amber, encaging molecules and membranes the way amber traps insects. Although many relevant biophysical and biological aspects have been reported,[6–14] convincing molecular explanation for this natural action has not been offered. In the attempt to search for the 'missing link' which makes trehalose action so special, we present here some ideas which add a new perspective to the overviews which have already appeared in the literature. In particular, the transformation path of trehalose from the solution to the solid state is considered to be important for the interplay of water with trehalose in living organisms.

In order to pursue this aim, some thermodynamic properties of the trehalose–water system will be reviewed, together with the current hypotheses of the mechanism of trehalose protection. Findings for trehalose polymorph transformations[15–17] will then be linked to the already known properties, to produce a possible explanation for the mechanism of anhydrobiotic action.

2. Are trehalose properties really anomalous?

The physico-chemical properties of trehalose are often described as 'anomalous', especially near the saturation or the glass transition lines. A summary of its characteristics is therefore necessary. The results presented here may serve in the formulation of the correct mechanism of protection ascribed to trehalose properties.

Solution properties.—Some thermodynamic data on the trehalose–water system (Fig. 1) can be found in the literature, although most of the solution studies have dealt with physical properties related to conformational,[18–28] and structural and dynamical aspects.[29–39] Thermophysical properties of aqueous trehalose solutions have been reported by Miller et al.,[40] mainly with the intention of providing data for their supplemented phase diagram. Solubility data from several laboratories are collected in the review paper of Chen et al.,[41] together with the freezing curve of trehalose–water solutions. Note that figure 1 of Ref. 41 shows uncorrected data, since the freezing line of water is not matched with the definition temperature of ice at 0 °C (273.15 K), but it is shifted to about 10 °C (ca. 283 K). It is apparent from these literature values that solubility data fall along two different lines with the curve given by Miller et al.[40] and Mehl[42] situated at lower concentration of trehalose with respect to the solubility curve given by Nicolajsen and Hvidt[43] and by Green and Angell.[44] The question of whether two different crystalline hydrated forms exist as equilibrium species has been raised, but without any at-

tempt to answer the problem. Among the other thermodynamic data of concern are the heat of solution of trehalose, both crystalline dihydrate and amorphous,[39,40] the activity coefficient,[45] and the heats of dilution.[46,47]

However, the thermodynamic significance of solubility lines has not been discussed in detail for correlation with other properties that are also known. Indeed, knowledge of the whole thermodynamic architecture must fit in a unique 'joint', with all the state functions related to each other. In fact, literature data are sufficient to formulate an analysis of the thermodynamic congruence on the basis of the relationship between solubility and heat of solution data of non-ideal aqueous solutions.[48] The correct thermodynamic equation which takes into account the non-ideality of the saturated solution of non-electrolyte mixtures, with a molality m and an activity coefficient γ, is the following

$$\Delta_{DS}H = \Delta_{IS}H - \Delta_{DIL}H = -R\left[d\ln m/d\left(\frac{1}{T}\right)\right]_{sat}\left[1 + \left(\frac{d\ln\gamma}{d\ln m}\right)_{sat}\right]_T \quad (1)$$

where the subscripts DS, IS and DIL refer to the 'differential' and 'integral' heat of solution and the heat of 'dilution', respectively. Only for ideal solutions are both the activity coefficient term and the heat of dilution term zero; in this simplistic case, equality between the two heats of solution with the heat of fusion and with the solubility derivative holds.[49]

The above equality can be checked for trehalose. The solubility concentration is taken as $m = 1.775$ mol kg^{-1} at 25 °C[40] and the enthalpy change is -1.24 ± 0.10 kJ mol^{-1} for the dilution from $m = 1.775$ mol kg^{-1} to $m = 0$.[46,47] Thus the last term on the right in Eq. (1), calculated from solubility[40] and activity data,[45] gives 19.4 kJ mol^{-1} at 25 °C. This value differs only slightly from that obtained from calorimetric data, that is, the difference $\Delta_{IS}H - \Delta_{DIL}H = 21.5$ kJ mol^{-1} ($\Delta_{IS}H = 20.3$[40] and $\Delta_{DIL}H = -1.24$ kJ mol^{-1}, respectively). However, the discrepancy becomes larger if the curvature of the $d\ln m/d\ (1/T)$ term is taken into account, a fact which may imply a change in the actual crystalline form in equilibrium with the saturated solution at higher temperatures.

The original paper by Green and Angell[44] reports a state diagram of trehalose–water system with two eutectic points at low (about

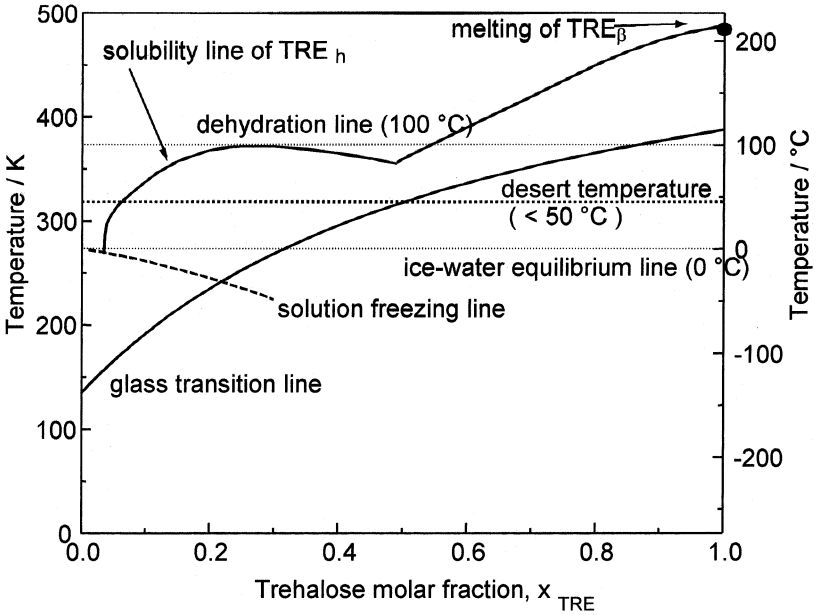

Fig. 1. The water–trehalose temperature–composition phase diagram. The composition is expressed as trehalose molar fraction, x_{TRE}. The transition lines define the stability of the dihydrate, TRE_h, and anhydrous, TRE_β, crystals and that of the glassy state, in addition to that of water freezing from the solution. For visual purposes, the relevant temperatures of 0, 50 and 100 °C are marked. The stability curves are calculated form literature data (see text).

$x_{TRE} = 0.1$) and high (about $x_{TRE} = 0.5$) trehalose concentration (see figure 2 of Ref. 44). Separation of ice and crystalline dihydrate trehalose occurs at the first eutectic point, while separation of crystalline dihydrate and crystalline anhydrous trehalose is reached at $x_{TRE} = 0.5$. Thus, the existence of an incongruent solid compound corresponding to a monohydrate form cannot be excluded, and has already been postulated.[40]

As a further check on the correctness of the calorimetric data, the heats of solution of TRE_h and amorphous TRE_{am} can be used to calculate the heat of transition for the reaction from TRE_h to TRE_{am} ($+2\ H_2O_{liquid}$). In addition to the $\Delta_{IS}H = +20.3$ kJ mol^{-1} for TRE_h, the literatures gives a $\Delta_{IS}H = -25.1$ kJ mol^{-1} for TRE_{am}.[40] Therefore, the transition from TRE_h to TRE_{am} ($+2\ H_2O_{liquid}$) requires 45.4 kJ mol^{-1} at 25 °C, which is slightly higher than that evaluated from direct DSC data.[17]

In conclusion, trehalose solution properties need to be explored more thoroughly, in order to verify the existence of other forms in equilibrium with the saturated solution and to appropriately relate all the stability regions in the state diagram. In particular, it is not clear whether the solubility discordance, for example, is a consequence of a mere kinetic effect or of the presence of a metastable hydrate form.

Glass transition.—On the basis of the thermodynamic behavior of concentrated solutions of sugars, it has been suggested that these systems easily form glasses when dried under suitable conditions at low temperatures, and that the glassy state is therefore involved in anhydrobiosis.[44,50–52] However, it can be seen that there is no evidence of differences in the physical properties of trehalose in comparison with other disaccharides in the solution state, nor in the amorphous state, apart from minor differences in the glass-transition temperature–composition profile. Data on the glass transition temperatures of trehalose[15,50–57] and on the effect of water plasticization on the glassy–amorphous carbohydrate properties[58–60] have been repeatedly reported and reviewed in the literature. Only recently, among all available data, a reasonably accredited value of the glass transition temperature has been defined.[17,41] The scattering of values of the glass transition temperatures for sugars (when data are available) is surely too large and beyond the expected experimental errors. This issue has already been addressed for the simple and stable sucrose molecule[61] and deserves an accurate and critical selection of literature findings.

It should be noted that the glass-transition temperature, T_g, of trehalose, which is about 120 °C, is comparable with that of a tetrasaccharide (e.g. maltotetraose) and is the highest in the disaccharide series, whose T_gs range mostly between 65 and 100 °C. However, an aleatory glass-like transition endotherm around 75 °C is often observed in the preparation of anhydrous trehalose, although it is not clear whether this glassy state may contain a small but constant amount of water. In other words, it is conceivable that under these conditions a segregated mixture of plasticized trehalose is formed.

The majority of the data are on the dependence of the glass transition temperature with composition. The most recent review of such data is given by the paper of Chen et al.,[41] where a fit of all experimental data provided the value of $k = 5.2$ for the unknown parameter of the Gordon–Taylor equation:[62]

$$T_g = (w_1 T_{g1} + k w_2 T_{g2})/(w_1 + k w_2) \quad (2)$$

where w_1, w_2, T_{g1} and T_{g2} are the mass weights and the glass transition temperatures of sugar (1) and water (2), respectively. Conversion to mole fractions is more suitable for the thermodynamics of mixtures of low-molecular weight molecules like the present case. For trehalose, this is particularly convenient since the mole fraction avoids the confusion which can arise in the state diagram for the equilibria with the dihydrate trehalose form. The mole fraction equivalent of the Gordon–Taylor equation (Eq. (2)) becomes in the notation of Couchman and Karasz approximation:[63]

$$T_g = (x_1 T_{g1} + (\Delta C_{p1}/\Delta C_{p2}) x_2 T_{g2}) \times /(x_1 + (\Delta C_{p1}/\Delta C_{p2}) x_2) \quad (3)$$

where x_1, and ΔC_{p1} (x_2, and ΔC_{p2}) are, respectively, the mole fraction and specific heat capacity increment of the component 1 (and component 2). The value $\Delta C_{p1}/\Delta C_{p2} = 0.385$

obtained as the fitting value of data in Fig. 1 with Eq. (3) can be accounted for using $\Delta C_p = 0.48$ J K g^{-1} for trehalose,[15] and 1.25 J K g^{-1} for water, which is within the range 1.05–1.39 J K g^{-1} indicated as the most reliable value.[64–66]

Diagrams like the one reported in Fig. 1 are 'state diagrams' which report not only the thermodynamic equilibrium lines between the different phases,[40,42,43,67,68] but also the 'non-equilibrium' lines that can be achieved in the experimental time-scale (usually of the order of minutes). More correctly, one should express these lines at definite value of the Deborah number (D_e) which gives the ratio between the relaxation time of the molecular system undergoing the transformation and the observation time of the phenomenon;[69] therefore, for the definition of the above non-equilibrium line, D_e should be always much greater than unity.

The concept of time-dependent structural changes is also implicit in the so-called fragility parameter, m, which has been devised as a distinctive property that characterizes dynamic relaxation at molecular level in the glass forming liquids.[70,71] The fragility is quantified by the dependence of relaxation times to temperature changes in the region approaching the glass transition. The fragility parameter was originally defined for physical properties associated with segmental relaxation dynamics (e.g. viscosity), but is currently evaluated from the slope of any relaxation time, plotted as a function of the reciprocal temperature, scaled by a reference temperature (i.e. m is a measure of an activation energy).[70] In practice, different methods give conceptually similar but not numerically equal values of the fragility parameters. For fragile liquids this parameter is definitely larger than for strong liquids; among all sugars, with a value of $m \approx 160–180$, trehalose falls in the range of fragile systems. A fragile liquid, such as trehalose and the trehalose–water system, shows a drop of several orders of magnitude in the viscosity values (from ca. 10^{14} to ca. 10^7 Pa).[37,40] Furthermore, there is evidence of an increase in fragility on passing from concentrate (low moisture) to dilute solution state.[37]

Crystalline state polymorphs.—The possible role of solid polymorphic forms that often exist for sugars[56,72] has not been investigated in details. In addition to the known stable dihydrate crystal form, TRE_h,[73,74] and to the anhydrous crystal form, TRE_β,[75] several transitions have been observed between polymorphic crystalline and amorphic species, within a complex interplay of water removal from the crystalline dihydrate matrix.[16,76] Other literature results have been concerned mainly with the thermogravimetric behavior of TRE_h crystals of different sizes.[76–78]

One form, TRE_α, can be obtained by dehydration of the TRE_h form at moderate temperatures (lower than 85 °C). The isothermal dehydration conditions can also be easily reproduced dynamically during non-isothermal calorimetric experiments, provided that low scanning rates are used and care is taken in order to remove the moisture from the sample. Indeed, the thermal characterization already reported by us[15] shows that anhydrous TRE_α can be formed by gently removing the water molecules under vacuum, that is, the rate of water loss does not allow the trehalose structure to relax into a more compact form. Only in this way can the original sugar architecture be maintained and the crystalline motifs be traced back to the organization of the trehalose molecules in the dihydrate form. Under these conditions, TRE_α can be fully re-hydrated back to TRE_h (Fig. 2), by exposing it to controlled humidity (for example at 50% relative humidity the rehydration process may take less than 24 h at 25 °C). Attempts to disclose the actual crystalline structure of TRE_α by using the Rietveld method on powder diffraction data already reported,[15] and from other diffraction data on small geminated crystals are under way.

Another form, TRE_γ, is produced at about 115 °C when a cold crystallization occurs after partial dehydration of TRE_h at a scan rate of about 12–20 K min^{-1}.[16] This latter form has been assessed by means of X-ray diffraction to be composed of two distinct domains, one made of TRE_h and the other made of TRE_β, conceivably with an external layer of anhydrous crystalline material surrounding an internal core of still hydrated crystals.

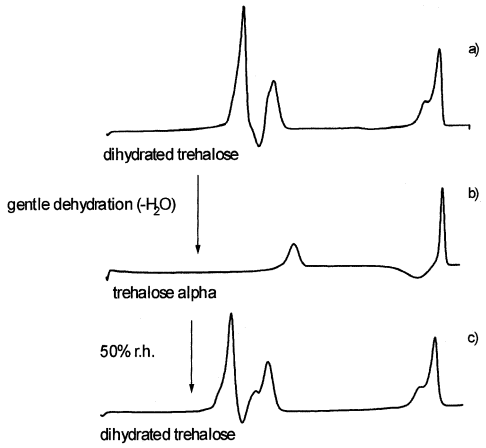

Fig. 2. DSC thermograms of (a): TRE_h, of (b): TRE_α obtained by gentle dehydration of TRE_h and of (c): the re-hydrated TRE_h obtained from TRE_α exposed to 50% relative humidity.

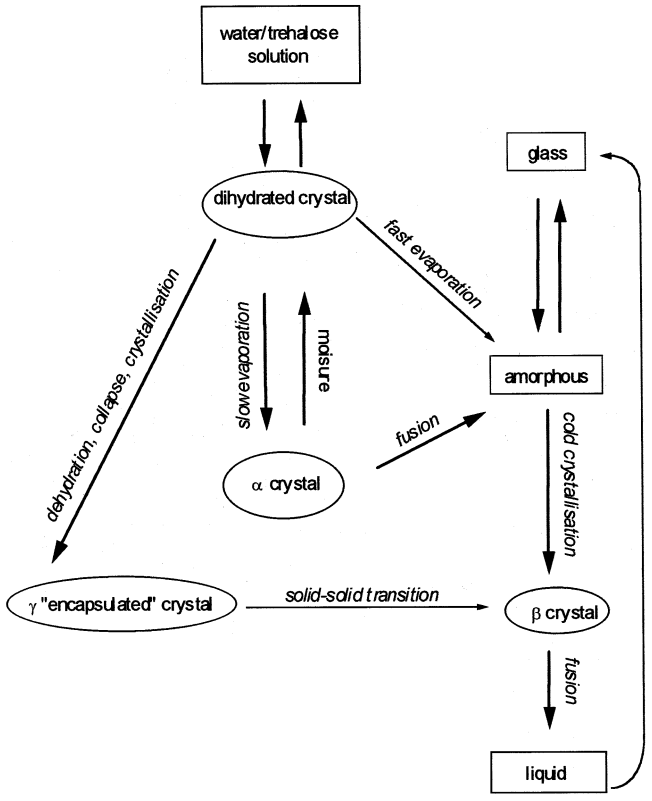

Fig. 3. Transformation path of trehalose forms. Dihydrate trehalose, TRE_h, is formed at equilibrium (reversibility) from the solution-state and can be transformed reversibly into TRE_α. In addition, depending upon the temperature and rate of water loss, TRE_h can give either an amorphous form or the TRE_γ form. All the transformations are described in the text and in Refs. 15–17.

The relevance of the time variable on the transformations has been emphasized[15] and more recently quantified by DSC analysis as a function of the scan rate.[17] In particular, from a detailed analysis of the dehydration process (see figure 1(b) of Ref. 17) it clearly emerges that different mechanisms occur in the range of scan rates explored (from 0.1 to 60 K min^{-1}). As a corollary to the structural transformations, it may be worth reporting here that small and wide angle X-ray powder diffraction studies using a SWAXS set-up with synchrotron radiation have clearly shown an extensive dissymmetry in the thermal expansion of TRE_h.

In addition to the above findings obtained in our laboratory on the several transformations among trehalose polymorphs, the possibility that trehalose (not fully dehydrated) could not only amorphisize but also crystallize has been demonstrated by Aldous et al.[79] This important observation not only demonstrates the ability of amorphous trehalose to crystallize, but also presents a strong argument in favor of the peculiar properties of trehalose. It has been argued that crystallization from a moist amorphous phase (that is the plasticized glass) generates a hydrate crystalline form. In this way water is removed from the amorphous phase, with an increase of its T_g, with the final results that the storage stability is further increased. The representation of all these transformations is given in Fig. 3, where the reversibility of transformation paths between the solution state, the crystalline TRE_h and the TRE_α forms is noteworthy.

Time-scale of transformations and relevance to the protective process.—An important point is the time-scale of the transformations reported above (see in particular the reversible paths in Fig. 3). Fast crystalline transformations (such as those originated from the devitrification of an undercooled liquid) are generally highly deleterious for the rapid change in volume and/or internal pressure. Slow transformations are far more important for the in-vivo processes, although they need accurate kinetic control and are therefore less suitable for 'putting principles into practice'.

Crystallization of plasticized trehalose (at $T > T_g$) has been achieved[79] by keeping the amorphous material for 12 h (overnight) at 82 °C (at $T < T_{trs}$ of transition of the dihydrate TRE_h form). Under these conditions, the time scale of the transformation may be

roughly calculated from the fragility curve of plasticized trehalose,[37] since this curve represents the rate of changes in viscosity in the range (and above) of the glass transition temperature.

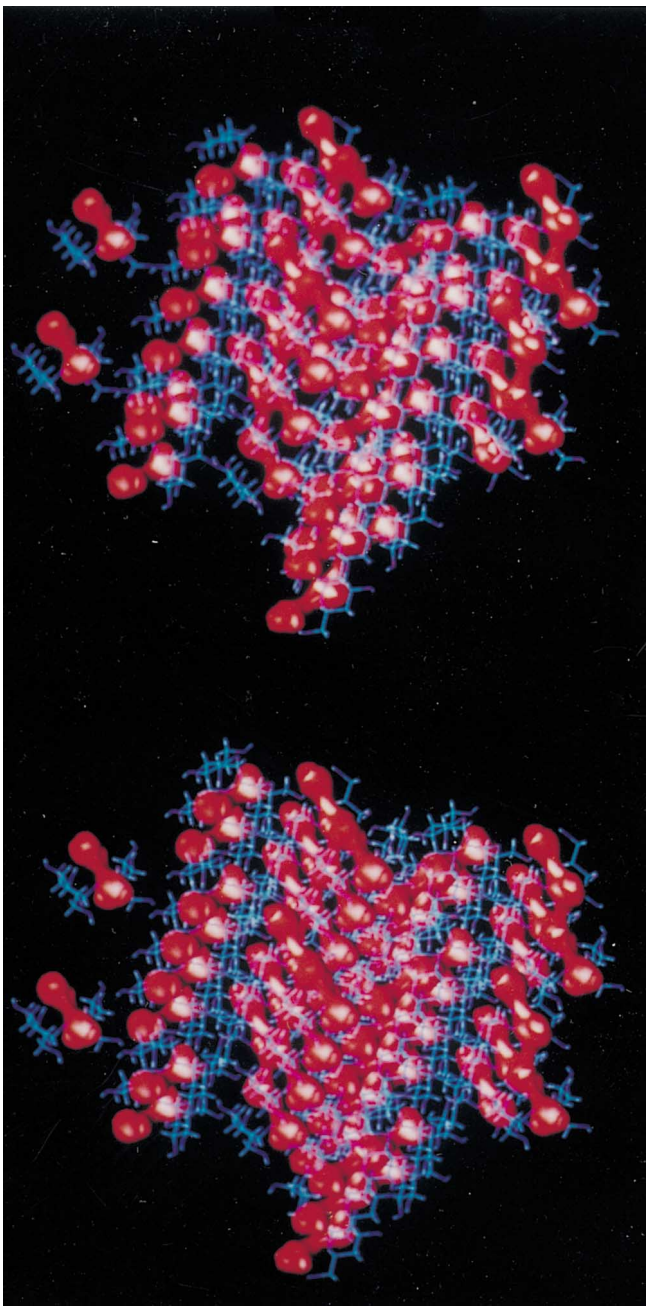

Fig. 4. Stereo view of a small fragment of the trehalose dihydrate (TRE_h) crystal. The sugar molecules are shown as stick figures in various shades of blue, to distinguish the three different atom types. The sites occupied by the water molecules are shown as red van der Waals surfaces, in order to emphasize the interconnected nature of the channels occupied by these water molecules. The probe radius used to calculate the surface was 1.4 Å, one half the optimum water–water separation distance.

Another point of interest is the time-scale of the crystallization of dihydrate trehalose from the solution. To the best of our knowledge, no data on this process are reported in the literature. However, some indirect observations come from the solubility and crystallization studies reported by Miller et al.[39] for the trehalose–NaCl–water system. The crystallization process is easier in mixed trehalose–salt solution, a fact that has been reproduced in our laboratory by growing dihydrate trehalose crystals at a trehalose concentration of 50% (w/w) in a 0.1 M NaCl aqueous solution. Therefore, although the pure trehalose–water system may appear more stable, in-vivo the presence of ionic low-molecular weight solutes and macromolecules is an effective induction–perturbation on the nucleation process. Under these circumstances, trehalose can easily crystallize on the ionic–polar hydrated biopolymer components with a process that is commonly called epitaxial crystallization.[80–82]

The dehydration–rehydration process is the third relevant point. In vitro, the measured time-scale of the dehydration process for trehalose is about 4 h at 85 °C[5] and longer than 24 h at 55 °C. Faster dehydration operated isothermally at higher temperatures or non-isothermally at scanning rates higher than about 1 K min^{-1} invariably produces other polymorphic states.[17]

The study of the polymorphic crystalline forms has led us to the observation that only TRE_α is able to easily capture water from the environment (Fig. 2). The formation of TRE_α and the reversible production of the dihydrate crystals by exposing the TRE_α form to humidity is believed to be a key mechanism for the protective action of trehalose, particularly since this reversible dehydration–hydration mechanism may gently occur in nature within the time scale of the anhydrobiotic protection. The dehydration process has been computer simulated by taking the crystalline features of the trehalose dihydrate crystal and removing the water molecules from their positions in the dihydrate crystal structure, where water molecules occupy adjacent positions along channels which lead to the crystal surface (Fig. 4). The rationale of this process is that water can escape from the lattice by hopping from site to site along these channels with an

activation energy that should be easy to compare with that calculated from non-isothermal thermogravimetric or calorimetric analysis. The extensive interconnections between sugar molecules in the lattice can energetically support the lattice structure even after the water removal. A residual stabilization energy of less than 10 kJ mol^{-1} has been found for the transition of the crystalline TRE_{α} form to the amorphous form at 125 °C.[15]

A summary of trehalose peculiarity.—The above critical survey of the physical chemical properties of trehalose reveals that this molecule is by no means particularly exceptional, despite the fact that it seems to possess each of the single peculiarities of the sugar class. For instance, to quote only the most relevant facts, among all disaccharides:

- trehalose is chemically stable (non-reducing terminals) like sucrose, while other sugars are not (e.g., maltose, lactose, …);
- trehalose forms both hydrate and anhydrous crystal polymorphs, like many of the sugar dimers, while sucrose does not;
- trehalose has the smallest partial molar volume in water, with a bent conformation like maltose, but apparently more restricted in the conformational space.

Whether the sum of all these particular characteristics, or some still hidden properties, confers to trehalose its hyped biological role has yet to be demonstrated, as will be shown below.

3. Survey of the bio-protective action of trehalose

Cell resuscitation has always been associated with the presence of endogenous trehalose produced by the organisms before entering the dormant state. Addition of exogenous trehalose has also induced similar results in cells not able to produce it spontaneously.[83] This observation implies a generalized protection by trehalose (and other sugars) and prompts scrutiny of the molecular properties which make this protective action so general and so efficient. The common mechanistic description of trehalose protective action is that trehalose acts at the molecular level, that is by forming 'molecular complexes' with the biomolecules. Several explanations have been proposed in the past and are only briefly reported below, while details and other related aspects are found in comprehensive reviews.[1,4,13,83–87]

The chemical stabilization hypothesis.—Although a chemical stabilization was earlier proposed in the 1970s, this type of action 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. Trehalose and sucrose, the sugars highly active in anhydrobiosis, are stable non-reducing carbohydrates and are hydrolyzed only at high temperatures, or in acidic conditions.[88,89] The stability of trehalose has probably still to be fully recognized in the 'physical' literature. Chemical reactivity, anomeric isomerization and conformational ring equilibria of reducing sugars in the solid state have to be reconsidered in view of the role that these molecular transformations may have in non ergodic transitions.[90–92] However, the absence of free reducing anomeric carbon in sucrose and trehalose is the main reason for ruling out a mechanism which could involve these reactions under physiological conditions.[93,94]

The water replacement hypothesis.—It has long been emphasized that the three-dimensional structure of biological macromolecules depends on the stabilizing effect of a water molecular layer which effectively interacts with surface residues via hydrogen bonding and electrostatic–polar interactions. It is believed 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 et al.[3] and recently other authors[95] 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'.[96] 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.[97] 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.[98] 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.[18,99] 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.

The glassy state 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 is therefore straightforward to suggest that this ability to form a glassy state[44,50,100] is relevant for the protective action of sugars in favor of anhydrobionts 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 (Fig. 1). Among all hypotheses, the formation of glassy layers of sugar molecules is now more widely credited from the physical point of view than the direct interaction of the sugar with protein and lipid surfaces.[100,101] Examples can be found showing a remarkable damping of protein dynamics when biomolecules are coated with trehalose anhydrous layers.[102–104] For this aspect, the high efficiency of trehalose has been ascribed to the high value of the glass transition temperature, an explanation which has been rejected however by other researchers in view of the minor effect of oligomers or dextrans with higher T_g values.[85] Furthermore, evidence has been offered in favor of the formation of trehalose hydrate crystals in the swollen lamellar structure of DPPC–sugar–water systems,[105] a fact which has been confirmed by our preliminary SWAXS experiments.

4. Conclusions

The literature experimental results, together with our new findings, can be rationalized in order to reconcile the several interpretative keys giving trehalose the greatest anhydrobiotic protective efficiency. The complete picture is based on the analysis of the several processes, and their time-scales, which must be similar to those likely to be encountered along the range of concentration changes and desert temperatures.

Considering the above reported evidences and including a few conceivable hypotheses (to be tested), the anhydrobiosis mechanism is proposed to be as follows: A biosynthetic trigger induces trehalose production in the metabolism of organisms exposed to high temperature and low humidity; then, the further decrease in water activity in the biological fluids induces nucleation and formation of layered epitaxial crystals of dihydrate trehalose on the surface of the cellular membranes, keeping water molecules in the same hydrogen bonding network as in the solvated trehalose. Water evaporation at desert temperatures (even up to 50 °C) and extremely low relative humidity increases the amount of dihydrate slowly formed at the membrane sur-

faces, without disrupting the native structure of the biological systems and capturing the residual water molecules. The closely packed layered crystals formed at low relative humidity undergo the final, slow dehydration, which eventually produces anhydrous trehalose which is mainly composed by TRE_{α}. Life functions would then be protected during dehydration as translational molecular motions and destructive chemical reactions are eliminated, preserving concurrently active molecular conformations and membrane structure. Macroscopically, the biological functions become 'frozen' without water crystallization. This life-safe protection is then guaranteed by the reversible paths (Fig. 3) between the solution state, the crystalline TRE_h and the TRE_{α} forms, all relevant for the in vivo role.

Studies on the persistence of the water–trehalose interaction from the solution state to the solid state, on the oriented (epitaxial) nucleation of trehalose on lipid surfaces and on the structure and stability of the new exotic crystalline phase are therefore necessary. Along this direction, crystallization and transitions of trehalose in the presence of a lipidic structure are being analyzed by several physico-chemical techniques (including small and wide X-ray scattering by synchrotron radiation) in order to provide new insights for the mechanism for the survival of biological functions by dehydration.

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