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The interplay of the rate of water removal in the dehydration of α,α-trehalose

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Abstract

The removal of water from α,α-trehalose dihydrate, T_h, has been studied both isothermally, at 130 °C, as a function of time, and as a function of the temperature at different scan rates by using differential scanning calorimetry. The protocols were chosen to discriminate the conditions that give rise to the formation of the anhydrous crystal form (T_β) and those that, according to previous findings, generate another form, T_γ, characterised by a different X-ray powder diffraction pattern. Scrutiny of the thermal properties of T_γ and of the carefully determined powder diffraction patterns discloses that T_γ is actually a mixture of the dihydrate and of the T_β forms. On the basis of the scanning rate under which the formation of the T_γ species occurs, the hypothesis is made that T_γ contains a T_h hydrated core encapsulated by a T_β anhydrous layer.

Keywords: α,α-Trehalose; Polymorphism; Dehydration; Calorimetry; X-ray powder diffraction

1. Introduction

α,α-Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a well-known non-reducing disaccharide that is present in many simple organisms (among others bacteria, yeast, fungi and several species of nematodes) where the concentration can be as high as 20% of the dry weight. It is capable of maintaining unaltered the integrity of the membranes during exposure to severe thermal stresses, either high or very low temperature [1,2]. The name α,α-trehalose [1] derives from a desert manna, trehala, in which the French chemist Berthelot found this sugar, even though Wiggers had already discovered it in rye ergot in 1832. When α,α-trehalose is synthesised in cells exposed to several stress conditions, the anhydrobiotic organisms are able to be dehydrated [3], kept in a dormant state for long periods of time (even up to 100 years) and rehydrated again to the original state.

Several hypotheses have been offered to explain the peculiarity of α,α-trehalose protection with respect to that of other sugars [2a]. The most credited current hypotheses are based on: (i) the water replacement, in which the interactions of the –OH groups of α,α-trehalose via hydrogen bonding with the phosphate head group are replaced by hydrogen bonding between the lipid and the bulk water [4]; (ii) the high glass transition temperature of α,α-trehalose, which leads to a glassy state

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that forms a cage surrounding the membranes, resulting in a reduction of the molecular motions preventing damage and preserving the life of the organism [5].

Due to the importance of α,α-trehalose in nature and the desire to have protective applications in biological products, this disaccharide has been studied from several points of view. A number of articles have appeared in literature on the crystal structure of the dihydrate and the beta anhydrous crystalline forms [6], while the molecular and conformational features have been studied by spectroscopic techniques [7–17] in the solid and solution states.

There are no doubts that the solid state stability of α,α-trehalose at different temperatures and relative-humidity conditions has to be fully known and taken into account for the assessment of its protection. Despite their underlying importance, the solid polymorphs have received very little attention [8,18–20]; α,α-trehalose has been studied in its crystalline dihydrate form (T_h) and in the anhydrous form (T_β). However, with the exception of the studies by Ding et al. [18a], Taylor et al. [18b] and Sussich et al. [20], much less is known about the pattern of polymorphic transformations and almost nothing is known about the structural features of other forms (either crystalline or amorphous). An anhydrous crystal form (T_α), reported for the first time by Perlin and co-workers [21], displays a different crystalline habit and melts around 130 °C. Further characterisation of this T_α form has recently been reported [20,22], together with the discovery of a new form, T_γ, with a transition point around 125 °C. If α,α-trehalose is involved in the preservation of organisms under thermal stresses, it seems obvious that a global knowledge of all the transformations is needed.

In order to further improve the understanding of α,α-trehalose metamorphism, we have systematically studied the thermodynamics and the X-ray diffraction pattern of the polymorphic transformations. This paper aims at the characterisation of some of these steps and at the knowledge of the complicated processes occurring in the solid dihydrate crystals when subjected to thermal stress. The final goal is to elucidate the mechanism behind the protection imparted by α,α-trehalose.

2. Experimental

Materials.—α,α-Trehalose dihydrate (T_h) was obtained from Sigma Chemical Co. and was used without further purification. The anhydrous form T_β was prepared according to the procedure of Perlin and co-workers [21] for their 'b' anhydrous form, keeping the dihydrate sample at 130 °C for 4 h. A second crystalline form, T_γ [20], was produced directly in the calorimeter by heating the α,α-trehalose dihydrate up to the water loss and by stopping the scan at the subsequent cold crystallisation, at a temperature around 115 °C; the specimen was then quenched to room temperature (rt).

Calorimetric measurements.—Calorimetric measurements were carried out with a Perkin–Elmer DSC 7 (power compensation) differential scanning calorimeter, connected to a computer via a TAC7/DX Thermal Analysis Instrument Controller. The 7 Series/UNIX DSC software by Perkin–Elmer was used for some of the measurements, while others were carried out with the Pyris Software 3.01 by Perkin–Elmer. The thermal unit was thermostatically controlled with an external thermocryostat in which the coolant was kept at 0 °C; a nitrogen flux was used as a purge gas for the furnace. Calibrations were made by using indium and zinc at the same scan rates used in the experiments. DSC scans were run on samples weighing between 5 and 15 mg and sealed in pierced aluminium Perkin–Elmer DSC pans; an empty aluminium pan was used as a reference.

X-ray diffraction.—X-ray diffraction patterns were obtained on powdered samples spread out on aluminium plates using a STOE-D500 X-ray diffractometer, at rt. Cu K_α radiation was obtained through a flat graphite crystal monochromator. Approximately 20 mg were loaded into the sample holder and scanned in the range 5–30° of 2θ with steps of 0.02° of 2θ with an acquisition time of 10 s for each step.

3. Results and discussion

Transformation of α,α-trehalose dihydrate to trehalose beta (T_β).—With the aim of understanding the process behind the formation of T_β and to study the $T_h \rightarrow T_\beta$ transformation, several vials containing the dihydrate material were put in an oven held at 130 °C and withdrawn after periods of incubation time (t_{inc}) ranging from 15 to 240 min. After cooling at ambient temperature, an amount from each vial was placed in a pierced aluminium pan and subjected to a DSC scan from 30 to 230 °C at a heating rate of 20 K min^{-1}. The first observation of the thermograms (Fig. 1) obtained for the different samples was that all the water of hydration has been removed from the original crystal structure at t_{inc} greater than 30 min; in fact, a flat baseline was recorded up to a temperature of 100 °C. However, a glass transition can be observed in the temperature range around 120 °C for samples incubated for short times.

The occurrence of a glassy state is clearly due to the fact that these samples are cooled to ambient temperature before the complete transformation to T_β and therefore the amorphous part in the specimen vitrifies upon cooling at about 130 °C. It should also be noted that the ΔC_p step for the transformation from the glassy state to the amorphous material decreases as a function of incubation time and tends to zero for samples with $t_{inc} > 100$ min (Fig. 2(a)). The absence of water in the amorphous/glassy sample was confirmed by the fact that the glass transition temperature (at $T_g =$ 120 °C, midpoint) was constant for all the samples. This is in agreement with the glass transition temperature already found for the transformation of anhydrous trehalose in the glassy state [20]. If traces of water were in the sample, then the glass transition would be at a much lower temperature; a decrease of about 10 °C is expected for less that 1% water for α,α-trehalose [23] and for sucrose [24]. The presence of an amorphous phase in the sample with $t_{inc} < 100$ min is also indicated by the presence of a small effect of cold crystallisation (Fig. 1), giving T_β just before its final melting endotherm. The values of the enthalpy associated with the cold crystallisation were found to decrease with increasing t_{inc} (Fig. 2(b)), while the enthalpy of melting changed only slightly and reached a limiting value of 52.6 kJ mol^{-1} for the samples kept for 4 h at 130 °C (Fig. 2(c)).

Another series of experiments has been carried out directly inside the calorimeter. α,α-

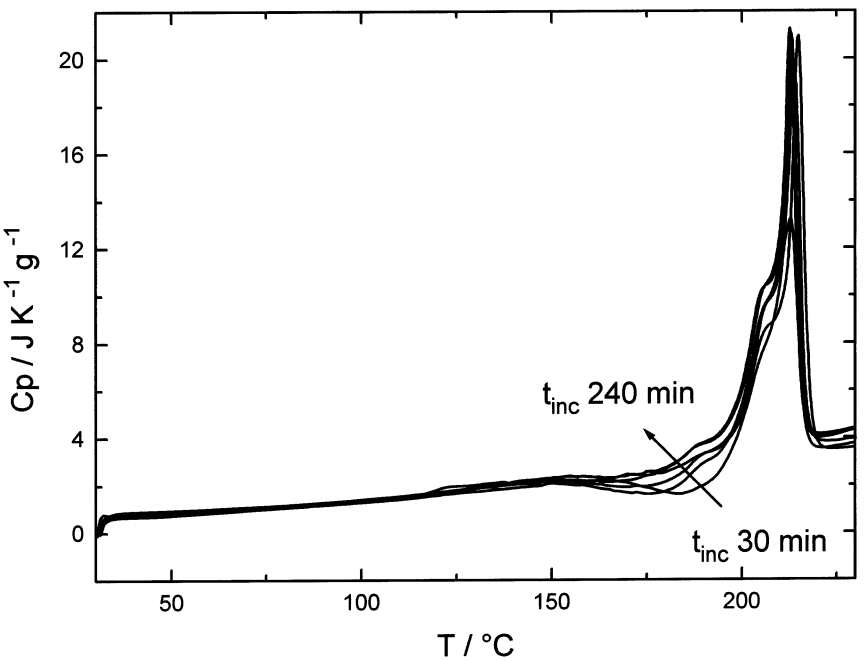

Fig. 1. DSC thermograms of T_β obtained from α,α-trehalose dihydrate (T_h) kept at 130 °C for different incubation times (t_{inc}); t_{inc}: 30, 60, 90, 120, 180, 200 and 240 min. A small ΔC_p step can be seen around 120 °C. Heating rate is 20 K min^{-1}.

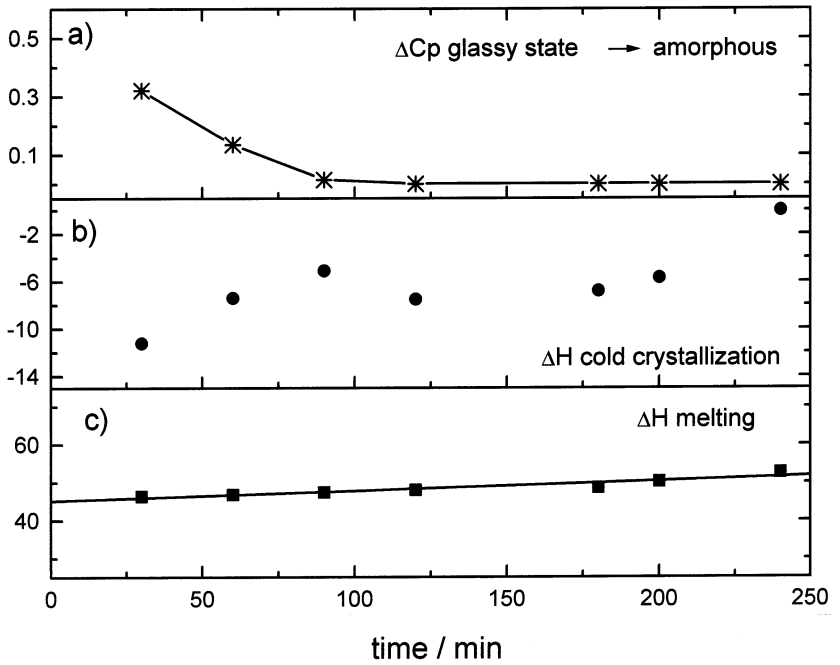

Fig. 2. Thermodynamic data for T_β obtained from T_h as a function of incubation time (t_{inc}): (a) ΔC_p for the transformation of the glassy state to the amorphous material for samples cooled to ambient temperature; (b) enthalpy for the cold crystallisation of the amorphous part into T_β; (c) enthalpies of melting of T_β, reaching the limiting value of 52.6. Units: ΔH in J g^{-1}; ΔC_p in J K^{-1} g^{-1}.

Trehalose was loaded into pans and placed in the calorimeter at 130 °C and then held for different periods of time. The following step in this procedure was a direct heating scan from 130 to 230 °C, without the cooling from 130 °C to rt. The resulting thermograms displayed a flat baseline and the only thermal event was the melting of T_β. The absence of a cooling step in the procedure prevented the formation of a glassy state and the only detectable signal was the melting of the crystalline portion formed inside the calorimeter. The absence of any small exothermic cold crystallisation suggests that the possible amorphous part could be steadily converted during the scan, even if the transformation to T_β was not completed.

Transformation of α,α-trehalose dihydrate to trehalose gamma (T_γ).—T_γ is the new form reported for the first time by Sussich et al. [20,22]. At that time, it was obtained by heating dihydrate crystals of α,α-trehalose at a scan rate of 20 K min^{-1} and stopping the scan at the cold crystallisation temperature after the water loss. A wide range of scan rates from 5 to 50 K min^{-1} has now been investigated for its preparation, stopping the heating scan after the dehydration and during the cold crystallisation. It appears that only the scan rates between 12 and 40 K min^{-1} result in T_γ being formed; by using scan rates below 12 K min^{-1} or above 40 K min^{-1}, the sample did not transform into T_γ. Below scan rates of 12 K min^{-1} the sample seemed to have enough time to slowly rearrange, giving an amorphous phase. By heating faster than 40 K min^{-1} there was not enough time for the cold crystallisation to occur and two broad overlapping peaks arose after the first endotherm [20].

In a previous paper, it was proposed that "A delicate kinetic balance exists between the water escaping from the crystalline structure and the structures collapse into the disordered state. Unless there is enough time or enough amorphous water still encapsulated to allow the trehalose molecules to become structurally reorganised, the water depletion may produce either an amorphous state or an unstable open cage water-free network" [20]. We can now support more convincingly this kinetic nature of the T_γ formation. At 12 K min^{-1}, the thermogram presents a very sharp peak of water loss; upon increasing the scan rate, the

curves become broader and shifted at slightly higher temperatures (Fig. 3). The thermograms of the T_γ forms, repeatedly prepared several times in this way and cooled to ambient temperature, are found to be coincident to each other and with that obtained by crystallisation at 20 K min^{-1}. However, it is worth noting that DSC and TGA on dehydrated α,α-trehalose give some slightly different results: DSC thermograms show the large thermal effect centred around 100 °C, while TGA experiments show the loss of water almost completed at 100 °C, with a small but continuous decrease in weight up to about 130 °C. The difference is clearly attributed to the fact that TGA and DSC cannot exactly reproduce the same experimental conditions, so that the rate of water loss is different; in DSC, the sample is encapsulated in a pierced pan, while in TGA experiments the sample is left open under a nitrogen flux.

The most extensive dehydration study of the α,α-trehalose dihydrate crystals is that of Taylor et al. [10,18b,c], which specifically addressed the relevance of the particle size to the kinetics of the transformation mostly at temperatures below 100 °C. They report, in their first paper, on the changes in the dehydration behaviour as a function of scanning rate (up to 10 °C min^{-1}) by TGA and DSC (up to 200 °C). While their results are in general agreement with our findings, unfortunately,

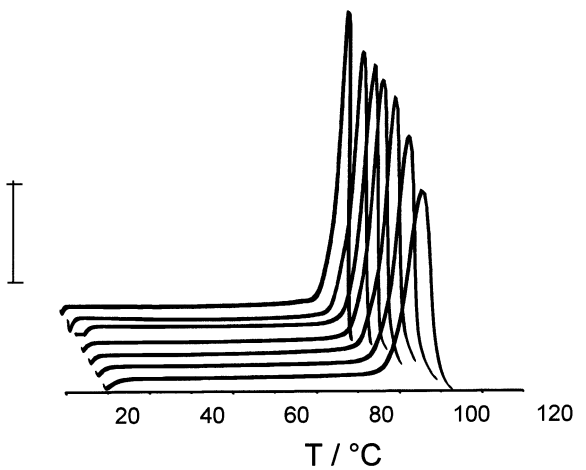

Fig. 3. Thermograms of T_h displaying the endotherm related to the water loss. The runs were stopped at the cold crystallisation leading to T_γ. Scanning rate are, from top to bottom: 12, 15, 20, 25, 30, 35 e 40 K min^{-1}. The C_p bar corresponds to 10 J K^{-1} g^{-1}.

they misassigned the phenomena underlying the thermal transitions, probably as a result of incorrect scaling of their thermal events as a function of the scanning rate. As a matter of fact, it is clear that the attention of the authors has been mainly drawn to the effect of the particle size and not to the actual transformations. In our case, the size of the crystalline dihydrate material ranged below about 100 μm, making our results more comparable with those obtained by Taylor et al. with the small–medium particles.

The question seems to be whether the preparation of T_γ can give a completely anhydrous form, even though the thermograms of T_γ do not show an enthalpic peak at about 100 °C that can be ascribed to the escape of water molecules. Given the operational and experimental differences between the two thermal techniques, we decided to rely only on DSC experiments, with an accurate monitoring of the weight changes of the samples at several stages of the scan. In particular, experiments have been designed to check traces of residual water in the sample after the cold crystallisation. Several runs were made on T_γ samples prepared inside the calorimeter at 20 K min^{-1}. The aluminium pans containing the freshly formed T_γ crystals were weighed prior to and after a heating scan from 30 up to 150 °C, the temperature at which T_γ had completely transformed. A small but not always reproducible difference in weight was found, providing the 'definitive' indication that some residual water is removed from the sample only at temperatures above 130 °C. The average result of these experiments is that about one water molecule per disaccharidic unit is removed around 100 °C during the first heating scan (20 K min^{-1}), while the second one remains trapped inside the crystalline material being formed. The hypothesis of the formation of a hemihydrate can immediately be borne out by the fact that the two water molecules have different properties and stabilities. This partial removal of the water is compatible with the crystal structure reported for the α,α-trehalose dihydrate, in which the two water molecules are not equivalent [6]. One water molecule is tetrahedrally coordinated, while the other shows a pyramidal co-

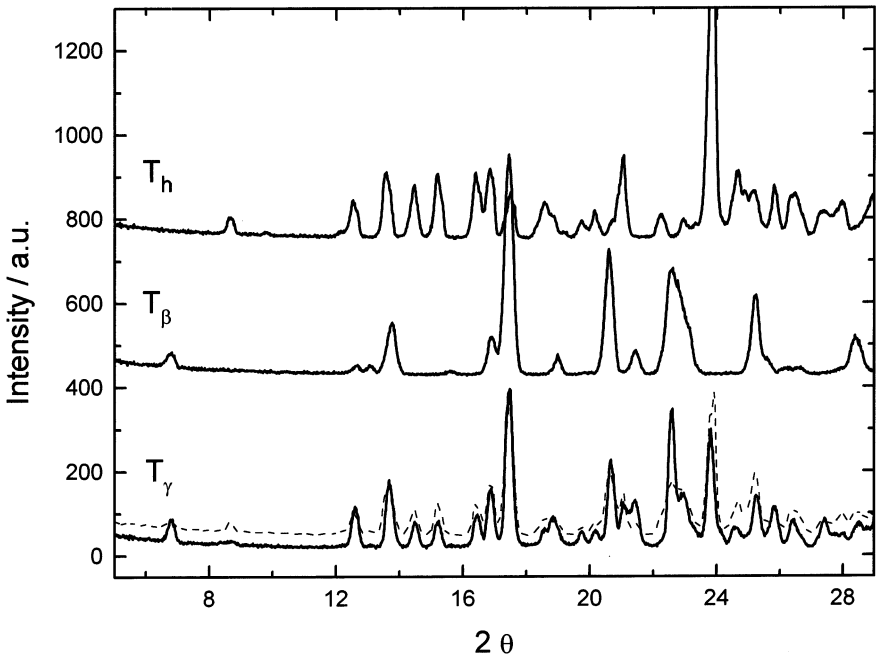

Fig. 4. X-ray powder diffraction pattern of T_h, T_β and T_γ (average over three different orientations of the specimen). The dotted curve is the sum of the powder diffraction of T_h and T_β, leading to the conclusion that T_γ is made up of two domains, one of T_h and the other of T_β.

ordination or an approximately planar trigonal arrangement, with a different number of hydrogen bonds, one having four hydrogen bonds with the glucose rings, the other only three bonds.

Characterisation of the structure of trehalose gamma (T_γ).—The above new findings at this point would have offered us an interpretation for the mechanism of formation of a transient hemihydrate structure, although a revision of our previous belief that the new form T_γ was indeed an anhydrous crystalline form also seems necessary. The solution of the structure of T_γ has therefore been considered mandatory. However, all the attempts to prepare suitable single-crystal specimens have been frustrated.

X-ray powder diffraction patterns (Fig. 4) have been recorded on T_γ samples. In order to have good diffraction patterns, the sample has to be ground to a fine powder; lateral mounting of the powder prevents a preferable orientation of the sample. However, the grinding of T_γ crystals leads to anomalous thermal behaviour and to a change in the crystal structure. This problem has been solved by exposing the flat disk obtained during the preparation of T_γ in the DSC pans, which is constituted of very small crystals, to the X-ray beam. Moreover, the final X-ray diffraction pattern was a mean of three X-ray diffraction patterns over the same disk with three different orientations.

By comparing the data obtained for T_γ with those for T_h and T_β, it was consistently found that the peaks of T_γ appear either in the pattern of T_h or in that of T_β. The interesting outcome is that the pattern of the gamma form is fitted by the sum of the two X-ray patterns of T_h and T_β. Therefore, T_γ seems to consist of two separated domains, one of T_β and the other of dihydrate molecules. This is in agreement with the DSC results, which show for T_γ the presence of water molecules. The presence of two domains, one anhydrous and the other hydrated, could explain why grinding of T_γ modifies the thermal behaviour: the heat effect associated with the friction presumably enhances thermal motions inside the sample and a small amount of water is free to escape from the crystals.

It is conceivable that the form called T_γ is indeed made possible by the partial removal of water with the formation of an external layer of anhydrous crystals with the core still made of α,α-trehalose dihydrate. The T_β layer constitutes a protective shield for the encapsulated α,α-trehalose dihydrate, provided that

the layer is thick enough to counterbalance the escaping tendency of the water molecules from the 'hydrate core' drops.

4. Conclusions

α,α-Trehalose dihydrate leads to several polymorphs depending on the thermal treatments to which it is subjected. By playing with temperature and time, the path can lead to anhydrous crystalline forms, T_β and T_α (which is the subject of a detailed investigation to be published), while a metastable, partially hydrated and not single-crystalline form, T_γ, is obtained by 'freezing' an ongoing transformation. The framework of the water–trehalose system is not simple, as there exist subtle interactions between water–trehalose and trehalose–trehalose. The tuning of the α,α-trehalose pattern of interactions to different temperatures could be the reason for the peculiar protective action. While the presence of a new single-crystalline form has been ruled out, the formation of T_γ is another indication of the kinetic interplay of molecular interactions in the trehalose–water system and potentially introduces another mechanism relevant to protection.

The findings of the present research have added another tile toward the understanding of the still puzzling phenomenon of trehalose action in anhydrobiosis. The work already carried out for the elucidation of T_α stability, its structure and the complete table of transformations of α,α-trehalose (to be published), will help in the understanding of the relevance of α,α-trehalose in cryoprotection.

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