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Carbohydrate Research 334 (2001) 233–241

CARBOHYDRATE RESEARCH

Infrared spectroscopic study on the properties of the anhydrous form II of trehalose. Implications for the functional mechanism of trehalose as a biostabilizer

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Received 16 April 2001; accepted 27 June 2001

Abstract

FTIR spectra were obtained for several different states of trehalose including dihydrate crystal, anhydrous form II (designated by Gil, A. M.; Belton, P. S.; Felix V. *Spectrochim. Acta* **1996**, *A52*, 1649–1659), anhydrate crystal, dried melt, amorphous solid and aqueous solution. From the observation of the symmetric and antisymmetric stretch vibrations of the glycosidic linkage, it is found that this sugar assumes at least three types of backbone conformations. Among them, the conformation with C_2 symmetry is characterized as 'open state', which means that the sugar easily absorbs water molecules. The conformation of the sugars in anhydrous form II and in freeze-dried trehalose is shown to be in the open state. Next, the hygroscopic properties of the anhydrate, form II and the amorphous solid are compared based on their IR spectra. Interestingly, form II alone is converted to the original dihydrate in a week under mild environmental-like conditions: relative humidity of 40% and room temperature. These results suggest the possibility that form II plays a role in avoiding the devitrification of the sugar glass. Finally, we discuss the role of form II in preserving freeze-dried biomaterials.

Keywords: Trehalose; FTIR; Anhydrobiosis; Metastable crystalline form; Vitrification

1. Introduction

In addition to providing nutrition to living organisms, many sugars perform other important functions that help to maintain or preserve life. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is one such sugar and functions as a water substitute in organisms that have been dehydrated due to exposure to an arid environment.[1] Many plants and insects that inhabit arid areas are observed to enter into a temporary death-like state when dehydrated and later revive themselves upon rehydration.[2] Trehalose appears to act as a substitute for water and thus maintains the life of such organisms.[1] Trehalose has no toxicity and is currently being studied for use in processed food,[2] in cosmetics, and for the storage of internal organs for transplantation.[3]

At least two hypotheses have been proposed to explain the molecular mechanism of desiccation tolerance induced by trehalose. One is the water replacement hypothesis[4,5] and the

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00182-3

other is the vitrification hypothesis.[6] Irrespective of which mechanism preferentially operates, it is of great importance to elucidate the reason why trehalose is superior in its preservation abilities under dry conditions.

Where carbohydrates are used as stabilizing excipients, say for proteins, their devitrification above T_g (glass-transition temperature) generally leads to rapid inactivation of the product.[6,7] However, if the carbohydrate forms a stoichiometric crystalline hydrate, a partial crystallization would prevent further devitirification by removing water from the amorphous phase and increasing T_g.[7] The crystallization of such a sugar hydrate provides additional desiccation by removing water from the amorphous state, thereby increasing T_g and the storage stability. This seems to be a generally accepted mechanism, explaining why the sugars that form stoichiometric hydrates act as good stabilizers. In the case of trehalose, the dihydrate crystal is formed when the amorphous solid with appropriate water content is kept above the glass transition temperature T_g.[7]

Crowe et al. compared the ability of trehalose and sucrose as a biostabilizer using the freeze-dried sample composed of trehalose (or sucrose) and liposome.[8] The rehydration behavior of these samples was examined by storing them at 40 °C and 58% relative humidity. Initially they took up similar amounts of water, but much of the absorbed water produced the dihydrate in the trehalose:liposome sample; whereas in the sucrose:liposome sample, the absorbed water resulted in a decreased temperature of the glass transition. The glass transition of the trehalose never fell below 65 °C, so the sugar remained in the glassy state, despite the sorption of water. These results seem to support the general mechanism mentioned above. However, if the storage temperature (40 °C) was always kept below T_g, they may not be straightforwardly interpreted. The enthalpy relaxation occurring below T_g might contribute to crystallization of the dihydrate.[9] Another possibility is that a component differing from the amorphous solid (sugar glass) was involved in that sample, and it was converted into the dihydrate by absorption of water.

It has been reported that trehalose exhibits complicated polymorphism depending on given thermodynamic conditions.[10–14] Currently, the polymorphic behavior of this sugar is not completely understood. Recent spectroscopic studies, including Raman, IR and NMR studies, have identified two anhydrous forms, designated as forms II and III.[15] Form III corresponds to well-known anhydrate crystal melting at 215 °C. Form II was obtained by leaving trehalose dihydrate crystals under vacuum for 48 h at 50 °C. Freeze-dried samples of trehalose alone and trehalose–lysozyme mixture give the Raman and IR spectra similar to those of form II.[15,16] This implies that form II is present in freeze-dried biomaterials. Therefore, it is of great importance to investigate the properties of form II.

In this report, from the observation of the symmetric and antisymmetric stretch vibrations of the glycosidic linkage, the sugar molecule in anhydrous form II is shown to take a conformation that is superior in the absorption of water molecules. Next, we compare the hygrosopic properties of form II, form III and the amorphous form. It is shown that form II is easily converted to the dihydrate crystalline form (form I) under mild environmental conditions: namely, a relative humidity of 40% and room temperature. On the basis of these findings, we will discuss the role of form II in the preservation of dehydrated materials.

2. Experimental

Trehalose dihydrate was purchased as the dihydrate from Wako Chemical Co., Tokyo. The dihydrate powder was pressed between two metal plates at 240 kg cm^{-2}. FTIR spectra were obtained for several samples differing in thermal and hygroscopic treatments. The pressed solid (sample A) was placed on KBr plates ($7 \times 7 \times 1$ mm), and its IR spectra were measured by a micro FTIR spectrometer. Another sample of the pressed solid, also placed on KBr plates, was set into a temperature controller (Mettler FP82-HT) placed on the X–Y stage of the micro FTIR spectrometer. Then, the sample was heated from 40 to 90 °C

at 1 °C min^{-1}. The resulting sample was denoted as sample B, whose IR spectrum was obtained with keeping the sample temperature at 90 °C. Similarly, sample C was obtained by heating the pressed solid from 40 to 160 °C at 1 °C min^{-1}, and its IR spectrum was measured at the final temperature. Immediately after the IR measurements, samples B and C were returned to rt, and subsequently hygroscopic treatment was carried out. They were stored for a week in a shielded box in which temperature and relative humidity were kept constant at 25 °C and 40%, respectively. The resulting samples are denoted as samples B′ and C′, whose IR spectra were measured at rt.

In addition to the above experiments starting from the dihydrate, we carried out comparative experiments for trehalose powder dehydrated in advance. First, trehalose dihydrate crystals, whose sizes were about 400 μm, were kept at 80 °C for 48 h and subsequently pressed in the same way as mentioned above. The resulting sample is denoted as sample D. This sample was also subjected to the same hygroscopic treatment as in the cases of samples B and C. FTIR measurements were performed for the resulting sample (sample D′) at rt.

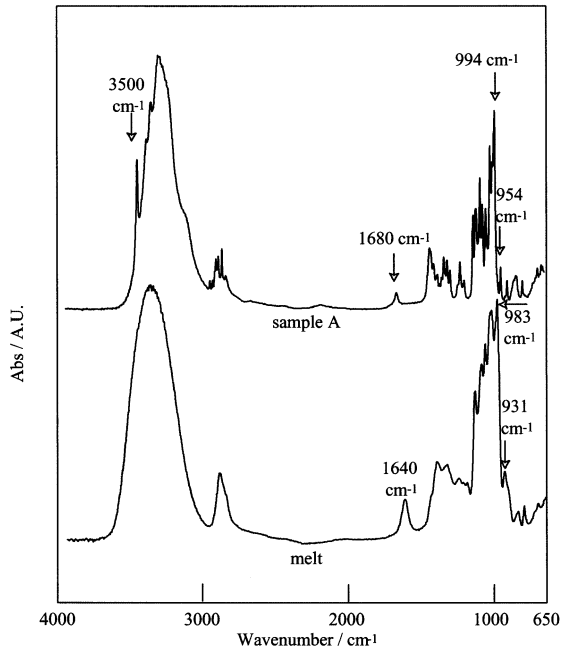

Fig. 1. IR spectra of sample A and the melt of trehalose dihydrate. Sample A is a pressed solid of the dihydrate powder that was measured at room temperature. The spectrum of the melt sample was obtained by heating a tablet of trehalose dihydrate–KBr mixture up to 160 °C.

In order to obtain the IR spectrum for the melt state of trehalose, we referred to the DSC (differential scanning calorimetry) study,[10] which indicated that trehalose is in the supercooled liquid state at a temperature range of 130–180 °C in a hermetically sealed pan. Here, we prepared a tablet of trehalose dihydrate–KBr mixture so as not to allow free evaporation of water from the sample, a condition similar to the case of a hermetically sealed pan in DSC. It was heated up to 160 °C and immediately followed by FTIR measurements.

The aqueous solution of trehalose (40% w/v) was placed between two CaF_2 plates (7 × 7 × 1 mm), and its IR spectrum was measured.

Except for the aqueous solution of trehalose, all the IR spectra were measured by using a micro FTIR spectrometer (JASCO MFT-2000) with a high-sensitivity mercury–cadmium telluride (MCT) detector. Thirty-two scans were performed for each measurement at a resolution of 4 cm^{-1}. During FTIR measurements, the sugar samples A, B, B′, C, C′, D and D′ were kept in the open-air state, in which free evaporation of water was allowed under the condition of uncontrolled humidity.

The IR spectrum of the aqueous solution of trehalose was measured using a FTIR spectrometer (JASCO FT/IR-680 plus). Thirty-two scans were performed for each measurement at a resolution of 4 cm^{-1}.

3. Results

Fig. 1 shows the IR spectra of sample A and melt trehalose. The former is in good agreement with literature data for trehalose dihydrate[15,16] and has some characteristic peaks at 3500, 1680, 994 and 954 cm^{-1}. According to the literature data,[17] the O–H stretch vibration of water with hydrogen bonding to other molecules appears at 3500 cm^{-1}. It is thus reasonable that the peak at 3500 cm^{-1} is assigned to the stretch vibration of the two crystal water molecules in the

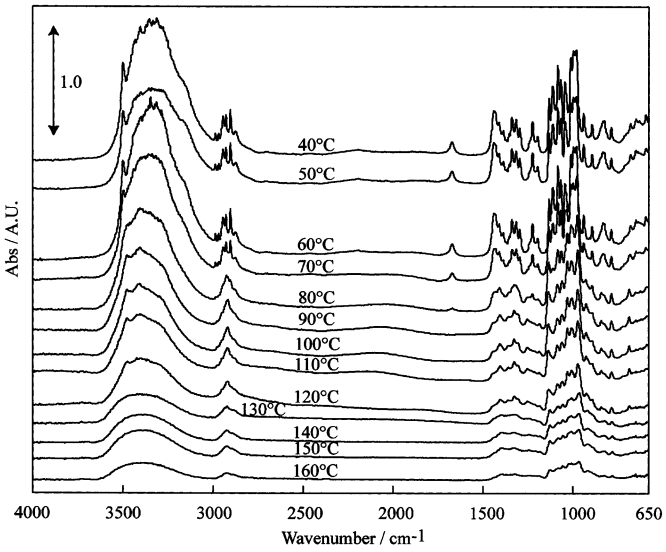

Fig. 2. IR spectra of trehalose dihydrate, measured from 40 to 160 °C at 10 °C intervals.

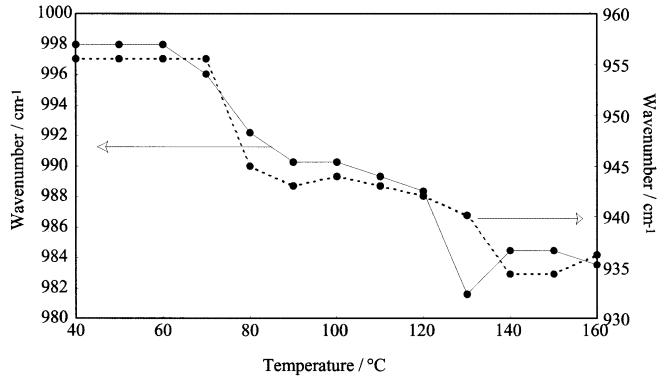

Fig. 3. Temperature dependence of the symmetric–antisymmetric stretch vibrations of the glycosidic linkage.

dihydrate sample. The peak near 1640–1700 cm^{-1} is assigned to the H–O–H bending motion and thereby uniquely appears in the IR spectrum of water.[18] Our previous FTIR study indicated that the bending peak of the crystal water in trehalose dihydrate appears at 1680 cm^{-1} at room temperature,[19] consistent with the spectrum of sample A. According to Ref. 20, the bridge C–O–C stretching modes are expected to occur between 1200 and 900 cm^{-1}. In addition, α-D-glucose does not exhibit any peaks between 914 and 998 cm^{-1}. Thus, the two peaks observed at 998 and 956 cm^{-1} may correspond to the two vibrational modes (antisymmetric and symmetric stretching) of the α-(1↔1)-glycosidic bond.

Fig. 2 shows the IR spectra of trehalose dihydrate, measured from 40 to 160 °C at 10 °C intervals. Interestingly, a significant amount of broadening resulted when the temperature passed around 80 and 130 °C. In addition, the peak of 1680 cm^{-1} assigned to the H–O–H bending disappeared after the temperature passed 80 °C. Fig. 3 shows the temperature dependence of the peak positions of the symmetric–antisymmetric stretch vibrations of the glycosidic linkage. Both peaks shifted in a step-wise manner to a lower wavenumber around 80 and 130 °C. These results suggest that the trehalose molecule undergoes structural transitions around 80 and 130 °C.

Fig. 4 shows the IR spectra of samples B and C. In these spectra, the peaks at 3500 cm^{-1} and 1680 cm^{-1}, observed in the spectrum of sample A, completely disappear. It is safely said that the water content of these samples is nearly zero. The peaks of the stretch vibrations of the glycosidic linkage slightly shift to a lower wavenumber (see Table 1). The spectrum of sample B is quite similar to that of form II (figure 4 in Ref. 15). The sole difference between the spectra of sample B and form II is that the latter exhibits

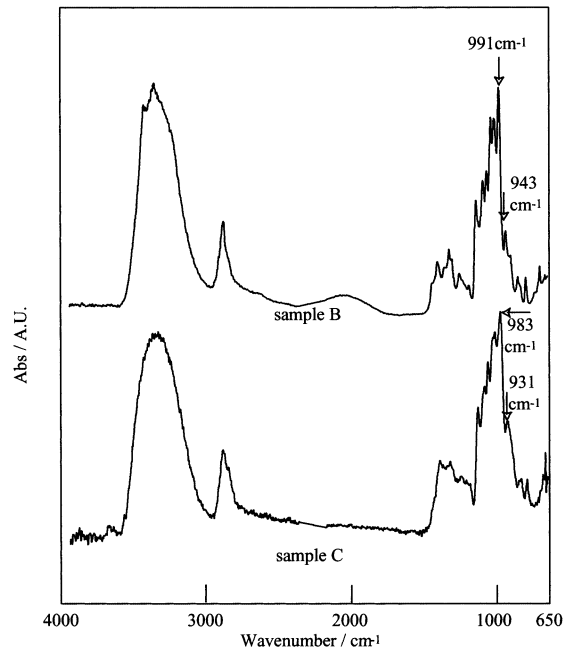

Fig. 4. IR spectra of samples B and C. Samples B and C were obtained by heating sample A from 40 to 90 °C or 40 to 160 °C at 1 °C min^{-1}, respectively.

Table 1
Some characteristic IR absorptions observed for the samples prepared under the different conditions

Sample	Water		Glycosidic bond	
	OH–O stretch	Bending	Symmetric–asymmetric stretch	
A	3500	1680	994	954
B			991	943
B′	3500	1680	998	956
C			983	931
C′		1640	983	931
D			986	943
D′			986	943
Freeze-dried [a]			991	941

[a] Sample obtained by freeze-drying the aqueous solution of trehalose.

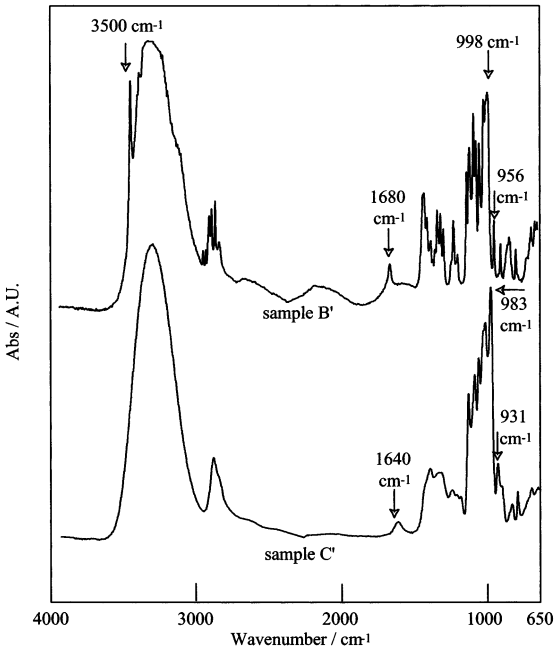

Fig. 5. IR spectra of samples B′ and sample C′. Samples B′ and C′ were obtained after storing samples B and C under the conditions of constant temperature (25 °C) and constant relative humidity (40%) for a week.

a small peak near 1680 cm^{-1}, clearly corresponding to the bending vibration of water. This indicates that the form II sample prepared in the previous study involves some amount of residual water. The spectrum of sample C is quite similar to that of the melt sample (Fig. 1), except for the fact that the latter spectrum, obtained for a tablet of trehalose–KBr mixture, still has the bending band of water. Thus, sample C is identified as dried melt.

Fig. 5 shows the IR spectra of samples B′ and C′ measured at room temperature. The spectrum of sample B′ is quite similar to that of sample A: the four characteristic peaks near 3500, 1680, 998 and 956 cm^{-1} are completely recovered after the hygroscopic treatment as described in the previous section. In contrast, the IR spectrum of sample C′ shows that all the peaks except for the bending vibration of water undergo no apparent change in comparison with the spectrum of sample C. Thus, sample C′ is identified as an amorphous solid. Considering the fact that the peak of the bending vibration of water appeared at 1640 cm^{-1} and the IR measurement was carried out at room temperature, the water observed is regarded as one adsorbed on the surface of sample C′.

Fig. 6 shows the IR spectra of samples D and D′. Similar to the cases of samples B and C, the characteristic peaks near 3500 and 1700–1600 cm^{-1} also disappear in the spectrum of sample D. This indicates that the

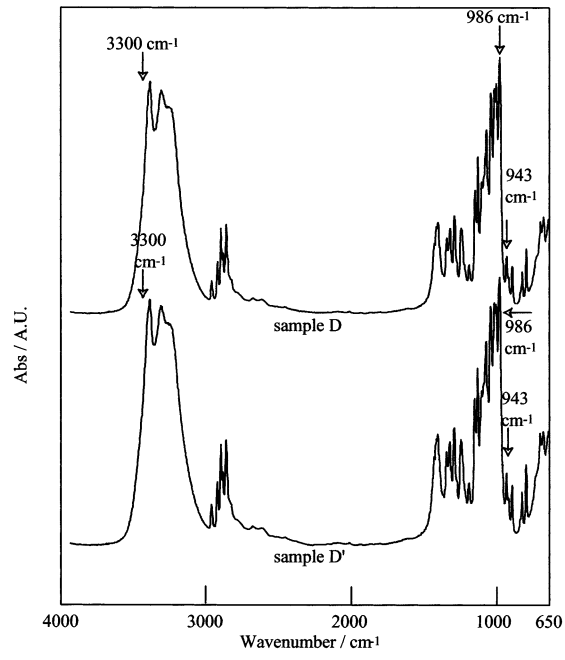

Fig. 6. IR spectra of samples D and D′. Sample D was obtained by dehydrating large size (400 μm) dihydrate crystals at 80 °C for 48 h. Sample D′ was obtained after storing sample D under the conditions of constant temperature (25 °C) and constant relative humidity (40%) for a week.

Table 2
Summary of the sample temperature, physical property and crystalline type for each of the samples studied

Sample	Temperature [a]	Property	Crystalline type [b]
A	rt	dihydrate crystal	form I
B	90 °C	anhydrous solid	form II
B′	rt	dihydrate crystal	form I
C	160 °C	melt	
C′	rt	amorphous solid	
D	rt	anhydrate crystal	form III
D′	rt	anhydrate crystal	form III

[a] Temperature at which the IR spectrum was measured.
[b] Classification according to Ref. 15.

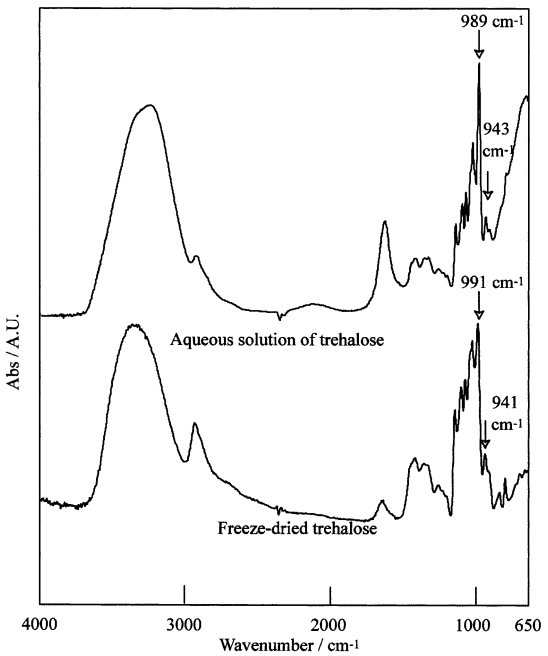

Fig. 7. Difference IR spectrum of trehalose in water and IR spectrum of freeze-dried trehalose. The difference IR spectrum of trehalose was obtained by subtracting the IR spectrum of water from that of the aqueous solution of trehalose (40% w/v).

crystal water has evaporated during the thermal treatment at 80 °C for 48 h. In addition, the peaks of the stretch vibrations of the glycosidic linkage slightly shift to a lower wavenumber (Table 1), and a new peak appears at 3300 cm^{-1}. As a whole, the spectrum of sample D is quite similar to that of the anhydrous form III given in figure 4 of Ref. 15. On going from samples D to D′, no apparent spectral change occurs. This implies that sample D does not absorb water under the hygroscopic conditions used here, which is in contrast to the cases of samples B.

Table 2 summarizes the sample temperature, physical properties and crystalline type assigned according to Ref. 15 for each of the samples studied.

Fig. 7 shows the difference IR spectrum obtained by subtracting the IR spectrum of water from that of the aqueous solution of trehalose. Together with this, the IR spectrum of freeze-dried trehalose is shown. In both spectra, the peak positions of the symmetric–antisymmetric C–O–C vibration agree with the corresponding peaks of sample B (Table 1).

4. Discussion

Our previous study indicated that the H–O–H bending peak of the dihydrate shifts steeply to 1640 cm^{-1} at 70 °C,[19] corresponding to the wavenumber of the bending motion of liquid water. This means that the crystal water undergoes a thermal transition from ice-like to liquid-like states at 70 °C, which suggests that the evaporation of the crystal water begins below the melting temperature of the dihydrate. Removal of the crystal water would exert some influence on the conformation and motional states of the sugar molecule itself. This is evident from the occurrence of the steep spectral changes around 80 °C as shown in Figs. 2 and 3.

To understand the detail of such a structural change, we examined the relationship between the peak positions of the symmetric–antisymmetric stretch vibrations and the two torsional angles (φ, ψ) around the C–O–C glycosidic linkage for trehalose dihydrate,[21,22] trehalose anhydrate[23] and trehalose in water[24,25] (Table 3). According to the data for the anhydrate crystal and the aqueous solution, the trehalose molecules in these systems have C_2 symmetry about the glycosidic linkage, although the (φ, ψ) values reported for

the solution phase are dependent on the experimental methods used (Table 3). Interestingly, the peak positions of the symmetric–antisymmetric stretches of these trehalose molecules agree with each other, and deviate from those of sample A (dihydrate crystal). This means that the positions of these peaks sensitively reflect the difference in the conformation (or symmetry) around the glycosidic linkage. The positions of these characteristic peaks of sample B (form II) are in good agreement with those of trehalose anhydrate and trehalose in water, which suggests the similarity in the backbone conformation among the sugar molecules in these systems. On the other hand, the trehalose molecules in samples C and C′ are different in conformation from those in samples A, B and D.

Judging from the peak positions of the C–O–C vibrations, the conformation of the sugar molecule in sample B′ is close to that in sample A. Furthermore, the peak of 1680 cm^{-1} was recovered simultaneously as shown in Fig. 5. Thus, it is concluded that water in the atmosphere was trapped by sample B, resulting in the formation of the dihydrate.

It is quite reasonable to assume that the conformation of trehalose in aqueous solution is favorable for interacting with the surrounding water molecules. This is supported by our previous molecular dynamics simulation for the aqueous solution of trehalose.[26] That study has indicated that all the hydroxy groups of trehalose simultaneously act as proton donors and acceptors to form hydrogen bonds with the surrounding water molecules. In this regard, the conformational state occurring in aqueous solution is hereafter called the 'open state', in which the sugar groups are responsible for absorbing water molecules. According to Table 1, the sugar molecules in samples B and D are also in the open state. As described above, sample B was converted into the dihydrate (sample B′) after the hygroscopic treatment. This phenomenon is interpreted as follows. First, the sugar molecule in sample B absorbs water molecules by forming hydrogen bonds with each other, and subsequently the conformation is relaxed into that of sample A (the dihydrate) to tightly bind two water molecules alone. Such a conformational change may be similar to the so-called induced fit occurring in the binding process of a substrate to an enzyme. The conformation of the sugar molecule in the dihydrate is hereafter called the 'closed state'.

The sugar molecule in sample D is also in the open state, but the absorption of water was not observed during the hygroscopic treatment. This fact would be explained from the difference in the crystal structure between sample D (the anhydrate crystal) and the dihydrate. The former's crystal structure is monoclinic with unit cell dimensions of $a = 13.003$, $b = 8.252$, $c = 6.799$ and $\beta = 98.33$.[23] The latter one's crystal is orthorhombic with $a = 12.230$, $b = 17.890$, $c = 7.596$.[21,22] The b value greatly decreases on going from the dihydrate to the anhydrate, which implies that water can not easily enter into the anhydrate crystal due to the dense packing of the sugar molecules.

Table 1 indicates that the conformational states of the sugar molecules in samples C and C′ belong to neither the open state nor the closed one. As described in the previous section, sample C is a dried melt. Sample C was quickly cooled to room temperature and the resultant sample was subjected to the hygroscopic treatment. During the storage, the sample temperature was kept below the glass transition temperature ($T_g = 115$ °C) of dried amorphous trehalose.[27] Thus, sample C′ is re-

Table 3
The relationship between the dihedral angles about the glycosidic linkage and the peak positions of the characteristic stretch vibrations of the α-(1↔1)-glycosidic linkage

Sample	Dihedral angle (°)		IR peak position (cm^{-1})	
	φ	ψ	Symmetric–asymmetric stretch	
A	−41 [a]	−58 [a]	994	954
D	−60 [b]	−59 [b]	986	943
Aqueous solution	−60 [c]	−60 [c]	989	943
	−41 [d]	−41 [d]		

[a] Taken from Refs. 21 and 22.
[b] Taken from Ref. 23.
[c] Taken from Ref. 24.
[d] Taken from Ref. 25.

garded as an amorphous solid. One of the most interesting findings in this study is that the conformation of the sugar in the melt-quenched sample (C′) is different from that in the freeze-dried sample. The conformation of the sugar in the latter is in the open state, judging from the data for the C–O–C stretch vibrations (Fig. 7 and Table 1). This finding indicates that the conformational state of the sugar molecule in aqueous solution is memorized in the freeze-dried sample.

We consider that form II may contribute to the function of trehalose as a protectant against desiccation. If the form II exists in a freeze-dried biomaterial–trehalose mixture, water would be preferentially absorbed into form II, being converted into the dihydrate crystals, rather than into the trehalose glass. As a result, the T_g of the glass region would not be lowered, avoiding the lowering of the storage stability. As described in the introduction, Crowe et al. indicated that trehalose is superior in the preservation of freeze-dried liposome to sucrose owing to the formation of dihydrate, which occurred at the storage temperature below the T_g of the sample. Their results could be understood on the assumption of the existence of form II in the trehalose: liposome sample.

As described in the introduction, we expected from the Raman spectra reported by Belton and Gil[16] that form II might exist in freeze-dried samples. Certainly, a freeze-dried sample of lysozyme–trehalose mixture gives Raman bands rather similar to that of form II in the range of 1000–800 cm^{-1} (figure 5 in Ref. 16). However, this would not necessarily indicate the existence of form II, because the spectral similarity in that range merely reflects the conformational similarity around the glycosidic linkage as shown in Figs. 4 and 7. It is of great interest to study what conditions are required to generate form II in a freeze-drying process.

The IR spectroscopy is appropriate for observing the conformational state of the sugar molecules and the formation and collapse of hydrogen bonds. However, it provides no direct information about whether a given sample is in the crystalline state or not. Thus, we cannot judge from the IR data alone whether sample B (form II) is a type of crystal or not. According to the DSC studies of the polymorphism of trehalose, the anhydrous form designated as T_α exhibits an endothermic peak at 130 °C with a small enthalpy of fusion (5.8 kJ mol^{-1}).[12] The temperature dependence of the IR spectrum of the dihydrate shown in Figs. 2 and 3 indicated the occurrence of the steep spectral change around 130 °C. Considering the fact that the IR spectrum of samples of B was observed at 90 °C, it is possible to assume that sample B corresponds to the T_α crystal. The detailed structural analysis of sample B is under investigation now in our laboratory.

5. Conclusion

In this study, it was found that trehalose assumes at least three different conformations about the glycosidic linkage. Among them, the open state, found in form II, freeze-dried sample and the aqueous solution, is the most important, because the trehalose molecule in this state could easily absorb water molecules. Form II seems to be a unique solid phase that is easily converted to the dihydrate by absorption of water, and thereby contributes to the preservation of dried biomaterials.

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