Experimental and Theoretical Study on the Glassy States of Trehalose and Neotrehalose

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Among the naturally occurring gluco-disaccharides, trehalose has the highest glass transition temperature (Tg) and the largest activation energy of enthalpy relaxation in the glassy state. Here, to understand why trehalose has such unique properties, we carried out FT-IR measurements and molecular dynamics simulations for trehalose and neotrehalose, and analyzed the hydrogen bond properties of both sugars in the amorphous states. By comparing the results for trehalose and neotrehalose, we reached the following interpretation: in the trehalose glassy matrix sugar molecules would be homogeneously distributed, whereas in the neotrehalose glassy matrix sugar molecules tend to be distributed separately into densely packed regions and sparsely packed ones. It is concluded that in the trehalose glassy matrix the conformational uniformity of sugar molecules brings about better (homogeneous) molecular packing, which in turn leads to the high Tg and the stability of the glassy state. Ultimately, such a unique property of trehalose originates from the presence of α,α-1,1 glycosidic bond responsible for the conformational rigidity.

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INTRODUCTION

Trehalose is a good protectant against various environmental stresses in many organisms. For instance, a larva of Polypedilum vanderplanki accumulates a large amount of trehalose when it is desiccated and enters into anhydrobiosis, a state of undetectable metabolic activity. Recently our group demonstrated that the anhydrobiosis is maintained as a result of vitrification of the accumulated trehalose.

In a previous study, we found that among the naturally occurring gluco-disaccharide trehalose has the highest glass transition temperature (Tg) and the largest activation energy of enthalpy relaxation in the glassy state. This means that trehalose is easy to be vitrified and its glassy state is highly stable. These properties are favorable as a desiccation protectant for biological materials. Thus, to understand the molecular origin of such unique properties of trehalose is particularly important, not only for elucidation of the nature of the anhydrobiosis in P. vanderplanki but also for industrial and medicinal applications of this sugar as a desiccation protectant.

In order to address this question, we here performed FT-IR measurements and molecular dynamics (MD) simulations for the amorphous states of trehalose and neotrehalose. From comparison of the hydrogen bonding properties of both sugars in the glassy state, we explored the
reason why trehalose is more easily vitrified than neotrehalose.

**MATERIALS AND METHODS**

Samples for FT-IR measurements were prepared as follows. First, 10 mg of trehalose dihydrate (or neotrehalose monohydrate) was dissolved in 1 mL of water and subsequently 20 μL of this solution was dropped onto a BaF2 disk (1 cm diameter, 2 mm thickness). Next, the disk was moved onto a hot stage and dried at 90 °C for 30 min. The resultant dried sample was subjected to FT-IR measurements, where the peak position of the OH stretching band appearing around 3400 cm⁻¹ was recorded over a temperature range from 30 to 90 °C. All the FT-IR measurements were carried out with an IMV4000 spectrometer, JASCO, Japan.

Initial systems for the MD simulations were built using the Amorphous Cell Construction software implemented in Discovery Studio. First, 75 molecules of trehalose (neotrehalose) were arranged at random in a unit cell (30Å × 30Å × 30Å) with a density of 0.9 g/cm³ and such a system construction was repeated three times for each disaccharide, namely, three kinds of the initial structures were prepared for each sugar system. Second, the energy of each system was minimized using the steepest descent method. For the resulting initial states, isothermal and isobaric MD simulations were carried out at a pressure of 0.1 MPa using the GROMACS ver. 3.3.1 program. Except for atomic charges, all the force field parameters for the disaccharides were taken from the general AMBER force field (GAFF). The atomic charges were obtained from quantum chemical calculations. Lennard-Jones cutoff radius was set at 10 Å and the coulombic interactions were calculated by use of the Particle Mesh Ewald method.

The glassy state of each system was produced by lowering the temperature of the system in a stepwise manner. The starting temperature was 600 K and subsequently the temperature was lowered at a rate of 25K/ns to the final temperature 200 K. Each of the consecutive simulations was started from the final configuration of the preceding simulation. The simulation at each temperature ran for 2 ns with a step size of 1 fs. The trajectory and thermodynamic quantities were collected from the latter half of each 2 ns simulation.

**RESULTS AND DISCUSSION**

Fig. 1 shows the temperature dependence of the OH stretching vibration peaks of trehalose and neotrehalose, where the temperature was increased at a rate of 2 °C/min. As can be seen from Fig. 1, the plotted data for each sugar can be fitted by two lines. The cross point of these lines corresponds to the glass transition temperature (Tg). As a result, the Tg values were determined to be 385 and 372 K for trehalose and neotrehalose, respectively. These values are in good agreement with those determined from DSC measurements (Table 1).

![Fig. 1. Temperature dependence of the OH stretching vibration peaks of trehalose and neotrehalose in the amorphous states. ■: trehalose, ●: neotrehalose.](image)

A notable feature of Fig. 1 is that the data for
trehalose is, as a whole, shifted towards the high wavenumber side compared with those of neotrehalose. This suggests that the hydrogen bond (HB) strength in the trehalose glassy matrix is weaker than that in the neotrehalose glass.

Recently, Imamura et al. found that the degree of HB formation in sugar glass could be measured by observing the change in the OH peak position on going from the room temperature to the glass transition temperature: a lower degree of HB formation corresponds to a larger shift of the peak. Such a shift can be estimated from the slope of the line on the low temperature side in Fig. 1. As a result, the slope (0.25) for the trehalose data was slightly larger than that (0.23) for the neotrehalose, which also suggests a lower degree of HB formation in the former.

Theoretical glass transition temperature for each sugar system was determined from the position of the inflection point in the density versus temperature plot (Table 1). For both sugar systems, the theoretical values were higher than the corresponding experimental ones. In general, Tg changes depending on the cooling rate used and more rapid cooling results in higher Tg. The cooling rate adopted in our simulation was larger than that in the experiment. This is the reason why the calculated Tg values were higher than the experimental ones. Apart from the absolute values of Tg, the relative order of Tg between the two systems was reproduced well, indicating the validity of the present MD simulations. In what follows, we discuss the origin for which the Tg of trehalose is higher than neotrehalose.

To compare the above FT-IR data, we counted the total number of HBs, namely the sum of intra- and inter-molecular HBs in the unit cell, where HB was defined as OH⋯ O distance <3.4 Å and ∠OH O>120°. As a result, the HB number for the trehalose system is smaller than that for the neotrehalose by 0.2-0.5 over the whole temperature range, qualitatively consistent with the FT-IR results as mentioned above.

Next, we counted the intra- and inter-HBs separately. The results are shown in Fig. 2A and 2B, where for each sugar system the data obtained from the three different initial structures (see METHOD) are plotted separately. The number of the intramolecular HB is significantly larger in neotrehalose than in trehalose. In contrast, the difference in the number of intermolecular HB between the trehalose and neotrehalose systems is gradually small with a decrease in temperature and eventually the values for both sugar systems.

<table>
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<tr>
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<th>Tg /K</th>
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<th>MD</th>
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<tr>
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<td>385</td>
<td>390</td>
<td>445</td>
<td></td>
</tr>
<tr>
<td>neotrehalose</td>
<td>372</td>
<td>378</td>
<td>403</td>
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</table>

Table 1. Glass transition temperatures of trehalose and neotrehalose as determined by different methods.

Fig. 2. Temperature dependence of the number of the intra- and intermolecular HBs.
becomes nearly equal to each other when the temperature is fully lowered than Tg.

Another interesting feature of Fig. 2A and 2B is that irrespective of the temperature examined the plotted data points are largely scattering in the case of neotrehalose, in other words, the HB networks generated from the simulations exhibited significant variations depending on the initial structures used. In contrast, the data for trehalose is well converged. These results could be interpreted as follows. In the amorphous state of neotrehalose, sugar molecules tend to be inhomogeneously distributed, responsible for the scattering of the number of intermolecular HB (Fig. 2B) and additionally their conformations are diversely distributed probably to cause the scattering of the number of intramolecular HB. On the other hand, in the amorphous state of trehalose, sugar molecules seem to be homogeneously distributed with no significant conformational variations. In a previous study, we showed that trehalose has the single stable conformation around the glycosidic bond, while neotrehalose can take various conformations. This supports the above interpretation about the feature of Fig. 2A.

The interpretation about Fig. 2B is supported by the data for intermolecular HB occupancy defined as follows:

\[
\text{HB occupancy} = \frac{\text{total hydrogen bonding time}}{\text{total simulation time}}
\]

This parameter was evaluated for each of the intermolecular HBs formed in each sugar glass and subsequently all the HBs were ranked in the descending order of this parameter value. In Fig. 3, the HB occupancy is plotted against the rank number, where the evaluation of the occupancy parameter was carried out for the glassy state at the temperature of 50 K lower than Tg in both sugar systems. Interestingly, the HB occupancy for the neotrehalose glass is larger than that for the trehalose one in a high rank region from 1 to 600, but it steeply decreases. As compared with the case of neotrehalose, the HB occupancy for trehalose changes slower with a longer tail on the low rank side. The HB occupancy value is considered to be a measure of HB strength: the stronger (stable) the HB is, the higher the HB occupancy is. Therefore, the results of Fig. 3 suggest that in the trehalose glass the strength of HB bonds is diversely distributed ranging from high HB occupancy (strong HB) to low HB occupancy (weak HB), while in the neotrehalose glass, such diversity tend to be considerably reduced: extremely speaking the HBs involved are differentiated in a bipolar manner, namely highly strong and highly weak bonds. This implies that the neotrehalose glass is composed of densely packed regions and sparsely packed ones.

Combined with the results of Figs. 2 and 3, it is concluded that in the trehalose glassy matrix the conformational uniformity of sugar molecules brings about better molecular packing (homogeneous spatial distribution), which in turn leads to the high Tg and the stability of the glassy state. Ultimately, such a unique property of trehalose originates from the presence of α, α-1,1 glycosidic bond responsible for the conformational rigidity.

Fig. 3. Plot of the HB occupancy against the rank number

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