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Thermodynamic, hydration and structural characteristics of alpha,alpha-trehalose

Takao Furuki,[1] Kazuyuki Oku[2], Minoru Sakurai[1]

[1]Center for Biological Resources and Informatics, Tokyo Institute of Technology, B-62 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan, [2]Glycosciene Institute, Researach Center, Hayashibara Biochemical Laboratories, Inc.,675-1 Fujisaki, Okayama 702-8006, Japan

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1. ABSTRACT

A nonreducing disaccharide, alpha,alpha-trehalose, accumulates endogenously in diverse anhydrobiotic organisms in their dehydrating process or prior to their desiccation, being thought to have a protective function either as a water replacement molecule or as a vitrification agent in the dry state. Trehalose acts also as a protectant against physiological stress, including freezing, ethanol and oxidation. To elucidate the origin of these different functions of this sugar, it is necessary to obtain a deep insight into the physicochemical properties of trehalose at the molecular level. In this review, we focus our attention on the thermodynamic, hydration and structural properties of carbohydrates, and extract the characteristic feature of trehalose. On the basis of these findings, we subsequently discuss the underlying mechanism for protein stabilization by trehalose in solution and for its anitoxidant function on unsaturated fatty acids.

2. INTRODUCTION

Water is essential for life, but some organisms survive desiccation and the dry state for long periods during which metabolism and life processes come to a halt, but resume on rehydration. Desiccation tolerance, or anhydrobiosis (‘‘life without water’’), is found across diverse biological kingdoms, including plants and animals such as mushrooms, yeast, fungi and brine shrimp (1,2). A nonreducing disaccharide, alpha,alpha-trehalose (hereafter trehalose, Figure 1), accumulates in these anhydrobiotic organisms and is thought to have a protective function either as water replacement molecules or vitrification agents in the dry state (1-3). Trehalose acts also as a protectant against other environmental stresses, freezing (4,5), ethanol (6) and oxidation (7-9).

In the food and pharmaceutical industries, trehalose has been utilized as a stabilizer to preserve

Table 1. Glass transition temperatures of water-free disaccharides[1]

	Ref. 35 [2]	Ref. 23 [2]	Ref. 34 [2]	Ref. 36 [3]
Trehalose[4]	116.9	107	79	
Maltose	100.6	92	87	95
Cellobiose	108.1			
Isomaltose				78
Gentibiose	100.8			
Sucrose	73.4	67	62	70
Turanose	88.2		52	
Leucrose	89.5			
Palatinose	68.9			
Lactose	112.3	101	103	
Mellibiose	101.3	91		
Lacturose	94.2			

[1]Given in °C as the mid point of the temperature range over which the stepwise change occurred in the heat capacity. [2]Heating at 5 °C min^{-1}. [3]Heating at 10 °C min^{-1}. [4]alpha,alpha-isomer.

proteins and enzymes in the dry state (10-13). Recently human platelet was successfully freeze-dried with trehalose (14). In addition to the preservation of such dried biomaterials, this sugar acts a good stabilizer of proteins and membranes in the presence of bulk water. It has been found that trehalose is very effective in stabilizing labile proteins during exposure to high temperatures in solution (15-18). Our early ^{31}P NMR study indicated that trehalose stabilizes unilamellar liposome by increasing the packing density among the constitutive phospholipid molecules, leading to inhibition of the fusion of the liposome (19). A recent finding is that trehalose inhibits the aggregation of proteins associated with Huntington's and Alzheimer's diseases. Tanaka *et al.* reported that trehalose could be used to inhibit the aggregation of polyglutamine *in vivo* in a rat model for Huntington's disease (20) while an *in vivo* study by Liu *et al.* indicated that this sugar effectively inhibits the aggregation and neurotoxicity of beta-amyloid 40 and 42 (21). Many other studies have been reported focusing on the application of this sugar, including in medical and cosmetic uses (1,3).

As described above, trehalose acts as a water substitute and exhibits a protective effect during various biological stresses. Our goal is to understand why trehalose is superior to other saccharides as such a stress protectant, and why it behaves as a chemical chaperone. To address this problem, it is necessary to gain a deep insight into the physicochemical properties of trehalose at the molecular level. In this review, we first focus our attention on the thermodynamic, hydration and structural properties of carbohydrates, then extract the characteristic features of trehalose. On the basis of these findings, we discuss the underlying mechanism for protein stabilization by trehalose in solution and for its antioxidant function on unsaturated fatty acids. Thus, the major part of this review concerns the biological role of this sugar in aqueous solution. Readers who are interested in the preservation of dry materials by trehalose are referred to excellent reviews by Crowe *et al.* (1,3).

3. THERMODYNAMIC CHARACTERIZATION OF CARBOHYDRATES

Thermodynamic properties of aqueous carbohydrates have been studied for more than three decades, because of their academic and commercial importance in view of biological processing and storage accompanying water stress such as freezing and desiccation. In particular, the study of glassy property of carbohydrates has made considerable progress in the 1990s'. The glassy property of a given carbohydrate follows the state diagram as shown in Figure 2, where T_g', T_g and C_g' are the glass transition temperatures of the solution and the pure solute, and the solute concentration in the residual unfrozen matrix resulting from a freeze concentration. In Figure 2, $T_g'_{max}$ corresponds to the glass transition temperature at the maximally freeze-concentrated concentration $C_g'_{max}$ and these quantities are regarded as the intrinsic properties of a given carbohydrate. In general, to measure $T_g'_{max}$, isothermal annealing is required at a temperature above T_g' but below the melting point of a given aqueous carbohydrate (23). T_g' values obtained without such annealing were, however, in good agreement with the corresponding $T_g'_{max}$, although the observed values for disaccharides were slightly smaller (24). Therefore, T_g' can be used to characterize the glassy property of di- and oligo-saccharides instead of $T_g'_{max}$. On the other hand, the relationship between $C_g'_{max}$ and C_g' is not so simple. Here we explain it by taking an example of aqueous sucrose. By cooling an aqueous solution of sucrose, water is crystallized and then the amount of ice can be measured from the heat of fusion of ice, H_f. Usually, H_f decreases linearly with the initial sucrose concentration (24). Then C_g' was estimated by extrapolating the linear regression line to $H_f = 0$. The resulting C_g'value was 69 wt%, which is smaller than the maximally freeze-concentrated concentration, $C_g'_{max}$, up to ca. 80 wt%. (23,25,26). In general, C_g' evaluated in the above way corresponds to the threshold when neither ice formation on cooling nor melting of ice on heating was observed in differential scanning calorimetry (DSC) measurements (26-29), in other words, the minimum concentration of the carbohydrate necessary for keeping all water unfrozen in the aqueous solution.

To date, there has been growing consensus that the glassy property is one of the key factors that should be considered when developing a method for the effective preservation of biomaterials (30-34). Early studies in the 1990s' found that from aqueous mono- to polysaccharides, there is a close relationship between molecular weighs of carbohydrates and their glass transition temperatures T_g' (30-34). However, some exceptional and unexplainable facts were also found. For example, a large difference up to 40 °C was found even among disaccharides (35): the T_g s of anhydrous alpha,alpha-trehalose and sucrose are 115 °C and 75 °C, respectively (Table 1). Such a discrepancy

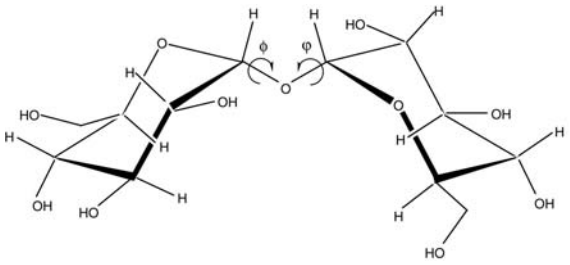

Figure 1. Molecular structure of alpha,alpha-trehalose.

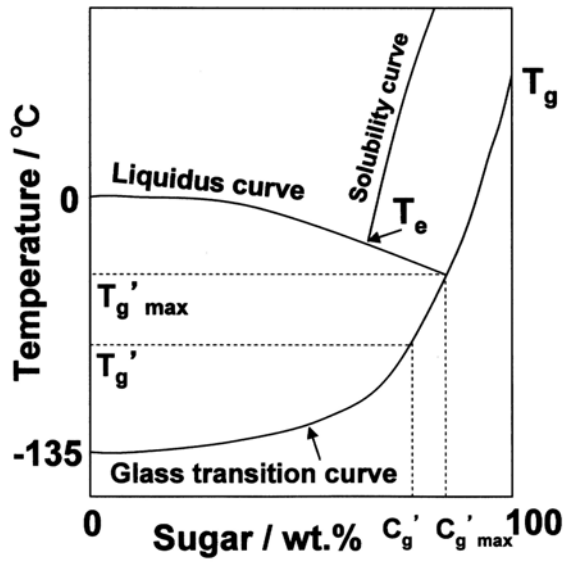

Figure 2. Schematic representation of the state diagram for the binary system, a carbohydrate and water. Unless the aqueous solution reaches its extremely freeze-concentrated state ($C_g'_{max}$) with the viscosity as high as 10^{12}~10^{14} Pa·sec, which prevents remarkably water molecules from diffusion and thereby halts ice crystal growth, the observed glass transition temperature T_g' is lower than $T_g'_{max}$. Usually the dissolved carbohydrate does not suffer from crystallization even at the eutectic point (T_e) because of its viscous propensity. The glass transition temperature of pure water, -135 °C, written on the ordinate was cited from ref.22.

should be rationalized by factors other than just molecular weight.

To address this problem, one of the authors of the present review (T. F.) investigated the thermodynamic properties of aqueous carbohydrates from mono- to oligosaccharides (Tables 2 and 3) through DSC measurements. Figure 3 shows the plot of T_g' vs. U_w, where U_w is the amount of unfrozen water (given in moles of water per mole of carbohydrate) calculated from the C_g' value at H_f = 0 obtained in the same way as described in the above paragraph. As a whole T_g' is mainly determined by the molecular weight of the carbohydrate dissolved in water, whereas U_w varies from sugar to sugar even among a family with the same molecular weight such as disaccharides. U_w is found, however, to increase linearly with T_g (Figure 4) (24,37). It is noteworthy that the following relationships exist between the molecular structure of carbohydrates and their thermodynamic properties: (1) for gluco-disaccharide (No. 1 to 11), T_g and U_w depends on both the position and type (alpha or beta) of the glycosidic linkage, (2) the disaccharides composed of glucose and fructose residues (No. 12 to 15) have a lower T_g and smaller U_w than other disaccharides, and (3) disaccharides with a galactosyl residue (No. 16 to 18) have a higher T_g and a larger U_w than other disaccharides.

Especially for trehalose, various T_g values have been reported so far from 73 °C (38) to 116.9 °C (35), although the value of 115±2 °C appears to have been widely accepted as the exact T_g of anhydrous trehalose (35, 39-41). The T_g of trehalose (numbered as 1) is highest among the saccharides shown in Figure 4, although the value is not necessarily special, at least not anomalous. In addition, trehalose has another noteworthy glass-forming property in favor of its application as a biological protective agent for long-term storage in dried states. Namely that glassy trehalose is more stable than other vitrified, well-known disaccharides, as demonstrated by an up to 150 kJ mol^{-1} larger activation energy of the enthalpy relaxation, ΔE_{rel}, for glassy trehalose relative to that of glassy maltose or sucrose (41). ΔE_{rel} is thought to be the activation energy of the translational diffusion of molecules forming the glass of interest, being a direct measure of the chemical and physical stability of the vitrified matrix. In summary, the characteristic physicochemical nature with not only high Tg but also high ΔE_{rel} makes trehalose a superior biological protective agent to other saccharides.

The dependence of unfrozen water content on carbohydrate species is closely related to the extent of forming or breaking hydrogen bonds with water. This view is supported by the correlation found between U_w and ΔH_{sol} (Figure 5), where ΔH_{sol} represents the heat of solution of amorphous carbohydrates and can be approximated as the sum of three pair-wise interactive contributions.

$$\Delta H_{sol} = \Delta H_{s\text{-}s} + \Delta H_{w\text{-}w} + \Delta H_{s\text{-}w} \qquad (1)$$

where $\Delta H_{s\text{-}s}$ represents the enthalpy required for breaking the inter- and in some cases intra-molecular interactions in the amorphous carbohydrate to be dissolved (i.e. $\Delta H_{s\text{-}s}>0$), $\Delta H_{w\text{-}w}$ the enthalpy associated with breaking hydrogen bonds among water molecules (i.e. $\Delta H_{w\text{-}w}>0$), and $\Delta H_{s\text{-}w}$ the enthalpy of forming hydrogen bonds between water and the dissolved carbohydrate (i.e. $\Delta H_{s\text{-}w}<0$). Unlike the amorphous carbohydrates, ΔH_{sol} for crystalline carbohydrates is positive (35,42), indicating that $\Delta H_{s\text{-}s}$ is larger in the latter than in the former. ΔH_{sol} is a good measure for the capability of hydrogen bond formation of solutes with water, under the assumption that the change of $\Delta H_{s\text{-}s}+\Delta H_{w\text{-}w}$ from sugar to sugar is negligible relative to that of their associated $\Delta H_{s\text{-}w}$. In Figure 5 the linear correlation holds true even in a narrow region, the middle region in Figure 5, to cover disaccharides. This suggests that the correlation originates from the difference in stereochemistry among the carbohydrates studied

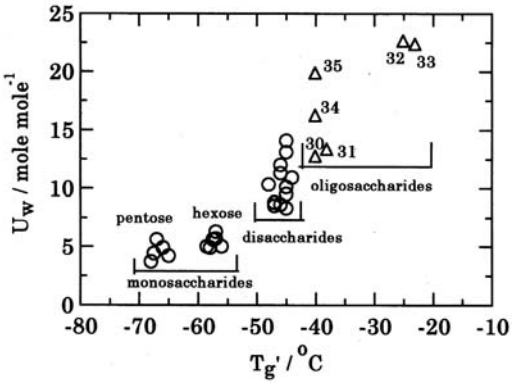

Figure 3. Plot of unfrozen water U_w vs. glass transition temperatures of aqueous carbohydrates T_g'. Numbering of oligosaccharides is given in Table 3. For mono- and oligosaccharides, numbering is omitted in this figure because of their crowded plots.

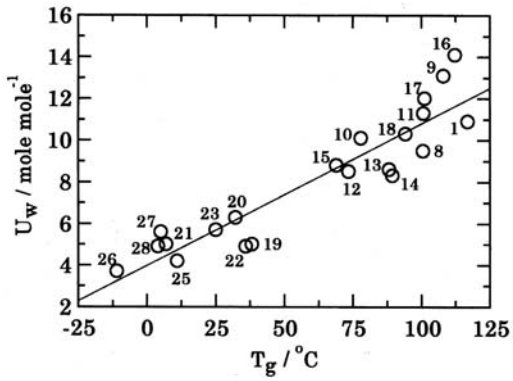

Figure 4. Plot of U_w vs. glass transition temperatures of water-free carbohydrates T_g. Numerical data for T_g were cited from refs. 23 and 35. U_w data were from refs. 24 and 37. Numbering is given in Tables 2 and 3.

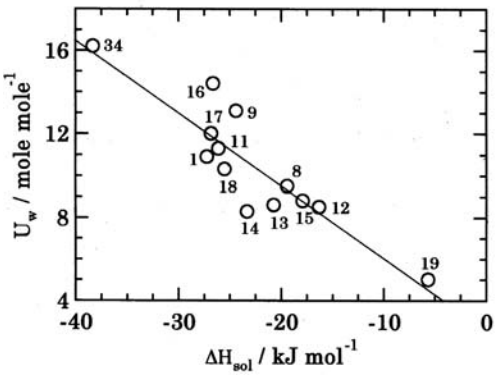

Figure 5. Plot of heat of solution ΔH_{sol} vs. U_w. Numerical data for ΔH_{sol} were cited from ref. 35.

4. HYDRATION STRUCTURE OF CARBOHYDRATES

A possible factor determining the unfrozen behavior of an aqueous solution of a dissolved solute is the compatibility of the tetrahedral hydrogen bond network of water with the stereochemistry of the solute (43,44). In the field of liquid physics, ultrasound velocity measurements have often been employed to obtain information on the hydration shell structure, providing the isentropic coefficients of compressibility, $-(1/v)(\partial V/\partial P)_s$, of the aqueous solution (β_s) and of pure water (β_w), respectively (45). If the solute molecule itself is incompressible, which would be true for small molecules under ordinary pressures, the isentropic *apparent* partial molar compressibility, $K_{S,2}$, of the solution is given as follows (46):

$$K_{S,2} = 1000 (\beta_s - \beta_w)/md + \beta_s V_c \qquad (2)$$

where m, d, and V_c represent the number of moles of solute per kilogram of water, the density of the solution, and the molar volume at the molarity of solute, respectively. The isentropic partial molar compressibility, $K°_{S,2}$, can be estimated by extrapolating the molarity, m, to zero, i.e. as the limiting value at an infinite dilution. The values of $K°_{S,2}$ reflect sensitively the characteristics of the hydration shell structure. When a dissolved solute disturbs or breaks the three dimensional hydrogen-bond network of water surrounding the solute, the water in the hydration shell becomes denser and less compressible than bulk water. This situation leads to a more negative value of $K°_{S,2}$. Figure 6 shows the correlation between U_w and $K°_{S,2}$ in the case of carbohydrates. U_w increases linearly with decreasing $K°_{S,2}$, which indicates that more water remained unfrozen in the presence of a carbohydrate that causes destructuring of the hydrogen bond network of water (24,37).

A similar argument was also made by other experimental and theoretical studies using techniques such as Raman (47-50) or inelastic scattering (49), and molecular dynamics simulation (50,51), where, for example, the relative spectral contribution from bulk and hydration water to the O-H stretching band was comparatively analyzed for the aqueous solutions of trehalose, maltose, and sucrose. Of particular interest, according to ref. 50, is that trehalose exerts a superior, destroying effect – relative to the others – on the hydrogen bond network of water at a sugar concentration above 30 wt%. Moreover, in ref. 51, statistics of cluster sizes made of water molecules via their hydrogen bonds was investigated and it was found that in aqueous trehalose the water cluster size was smaller than in aqueous maltose and sucrose when the concentration of sugar was more than 40 wt%. Based on these results, the remarkable cryoprotective effectiveness of trehalose as compared with other disaccharides was interpreted as due to its poorer fit into the tetrahedral hydrogen bond network of water, which should destroy the pure water cluster and thereby hinder the growth of ice (47-51).

Galema *et al.* proposed a hydration model for carbohydrates from a detailed inspection of their $K°_{S,2}$ data (46). One of the features of this model is that the hydration property of carbohydrates is governed by relative orientation of the next-nearest neighbor hydroxyl (OH) groups, such as those in positions 2 and 4 of the pyranose

Table 2. Numbering and molecular structures of disaccharides studied[1]

No	Name	Molecular Structure
		D-Glcp and D-Glcp
1	Trehalose	• Alpha-D-Glcp (1↔1)-alpha-D-Glcp
2	Isotrehalose	• Beta-D-Glcp (1↔1)-beta-D-Glcp
3	Neotrehalose	• Alpha-D-Glcp (1↔1)-beta-D-Glcp
4	Kojibiose	• Alpha-D-Glcp (1→2)-D-Glcp
5	Sophorose	• Beta-D-Glcp (1→2)-D-Glcp
6	Nigerose	• Alpha-D-Glcp (1→3)-D-Glcp
7	Larminarabise	• beta-D-Glcp (1→3)-D-Glcp
8	Maltose	• Alpha-D-Glcp (1→4)-D-Glcp
9	Cellobiose	• Beta- D-Glcp (1→4)-D-Glcp
10	Isomaltose	• Alpha-D-Glcp (1→6)-D-Glcp
11	Gentibiose	• Beta-D-Glcp (1→6)-D-Glcp
		D-Glcp and (D-Fruf or D-Fruc)
12	Sucrose	• Alpha-D-Glcp (1↔2)-beta-D-Fruf
13	Turanose	• Alpha-D-Glcp (1→3)-D-Frup
14	Leucrose	• Alpha-D-Glcp (1→5)-D-Frup
15	Palatinose	• Alpha-D-Glcp (1→6)-D-Fruf
		D-Galp and (D-Glcp or D-Fruf)
16	Lactose	• Beta-D-Galp (1→4)-D-Glcp
17	Melibiose	• Alpha-D-Galp (1→6)-D-Glcp
18	Lacturose	• Beta-D-Galp (1→4)-D-Fruf

[1]Glcp, Fruf, Frup, and Galp represent glucopyranose, fructofuranose, fructopyranose, and galactopyranose, respectively.

Table 3. Numbering of mono- and oligosaccharides studied[1]

	Monosaccharides			Oligosaccharides	
No	Hexose	No	Pentose	No	
19	D-glucose	25	D-xylose	30	maltotriose
20	D-galactose	26	D-ribose	31	maltotetraose
21	D-fructose	27	D-arabinose	32	maltopentaose
22	D-mannose	28	L-arabinose	33	maltohexaose
23	L-sorbose	29	D-lyxose	34	raffinose
24	D-talose			35	stachyose

[1]Numbering continues from that in Table 2.

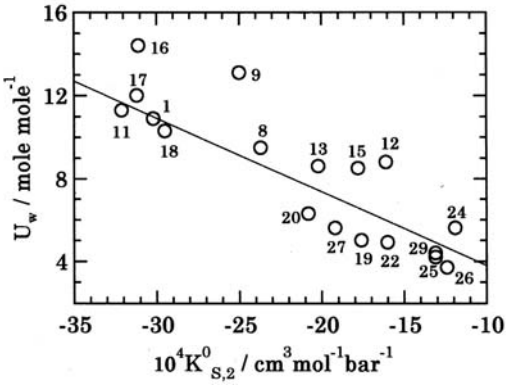

Figure 6. Plot of isentropic partial molar compressibility $K°_{S,2}$ vs. U_w. Numerical data for $K°_{S,2}$ were cited from ref. 46.

ring (Figure 7). When both of the two OH groups occupy the equatorial position of the ring in a chair conformation, the spacing of their oxygen atoms is ca. 4.85 Å, fitting well into the spacing between the next-nearest neighbor oxygen atoms of water molecules involved in the tetrahedral hydrogen bond network. From such a point of view, monosaccharides are classified into three groups. Group A: OH (2) is equatorial and OH (4) is axial; Group B: OH (2) is either axial or equatorial and OH (4) is equatorial; and Group C: both OH (2) and OH (4) are axial. For D-aldohexoses, D-galactose belongs to Group A, D-glucose and D-mannose to Group B, and D-talose to Group C. On the other hand, disaccharides are classified based upon the combination of their constitutive monosaccharides (Table 2). As far as we know, to date, there is little or no information confirming the conformational preference of carbohydrates in either freeze-concentrated matrix or anhydrous glassy states. However, in view of the good correlation between U_w and $K°_{S,2}$ (Figure 6), the stereospecific hydration model deduced from dilute aqueous solutions is recognized as a useful frame to rationalize the differences in U_w from sugar to sugar.

Table 4 shows the comparison between U_w and the hydration numbers determined by various methods for four representative disaccharides. Interestingly, U_w and the hydration numbers follow the same order in relative magnitude, lactose > trehalose > maltose > sucrose. Particularly it should be noted that the U_w and hydration number of lactose, composed of Group A and B monosaccharide units, are larger than those of trehalose and maltose, both of which involve only a Group B unit. This result indicates that the hydration properties of disaccharides are governed partially by the stereochemistry of the constitutive unit.

5. DYNAMIC ASPECT OF THE HYDRATION OF CARBOHYDRATES

In order to characterize the hydration phenomena in more detail, it is worthwhile to get information on the mobility of water molecules in the presence of carbohydrates. One of the useful techniques for such a purpose is ^{17}O-NMR (nuclear magnetic resonance) spectroscopy. In the so-called two state model (56), ^{17}O nuclei in the aqueous solution are assumed to be distributed between the following two motional states: the water in the hydration shell and the bulk water. If the exchange rate between hydration and bulk water is fast enough relative to the magnitude of the spin-lattice relaxation times of ^{17}O nucleus, the following relation is satisfied (56,57):

$$1/T_1 = (1-\chi_h)/ T_1^o + \chi_h/T_1^h \quad (3)$$

where T_1^o and T_1^h are the spin-lattice relaxation times of the natural abundance ^{17}O nucleus in pure water and in the aqueous solutions of carbohydrates, respectively and χ_h represents the mole fraction of hydration water. In the extreme narrowing conditions, the correlation time τ_c of molecular reorientation is obtained as follows (58):

$$1/T_1 = (3/125) (1+ \eta^2/3) (e^2qQ/h)^2\tau_c \quad (4)$$

where η and (e^2qQ/h) represent an asymmetric parameter of the electrical field gradient and the quadrupole coupling constant, respectively. The value of η is usually small,

Table 4. Comparison of numerical data of U_w with hydration numbers as determined by various techniques.

	U_w [1]	Hydration number [1]		
	DSC [2]	Viscosity and density [3]	QENS [4]	MD [5]
Lactose	14.4	8.3		
Trehalose	10.9	8.0	9.0	7.8 [6]
Maltose	9.5	7.5	8.4	
Sucrose	8.5	6.8	7.5	7.0 [7]

[1]Given in moles of water per mole carbohydrate. [2]The author (Furuki)'s work. Cited from ref. 24. [3]Cited from ref. 52. [4]Quasi-elastic neutron scattering. Cited from ref. 53. [5]Molecular dynamics simulations with the same force field (CHARMM) and water potential (TIP3P). The hydration numbers cited had been estimated as the averaged number of water molecules existing within 2.8 Å of the carbohydrate oxygen atoms throughout the simulation. [6]Cited from ref. 54. [7]Cited from ref. 55.

Table 5. Comparison of dynamic hydration characteristics of gluco-oligosaccharides[1]

	N_{DHN} [2]	τ_{ch}/τ_{co} [2]
Trehalose	48.3	7.08
Maltose	23.8	4.66
Maltotriose	35.6	5.64
Maltopentaose	71.0	5.39

[1]All numerical data were cited from ref. 59. [2]Details are described in the text.

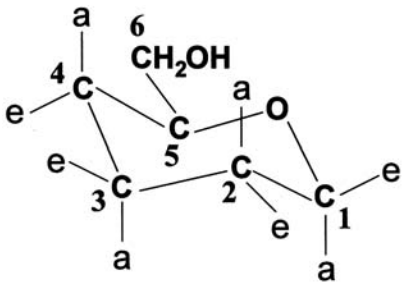

Figure 7. Schematic representation of the pyranose ring in a chair conformation, which aldohexoses usually adopt. The letters **a** and **e** indicate the axial and equatorial positions, respectively.

being omitted (58). The larger value of τ_c indicates more restricted molecular mobility. From eqs. (3) and (4),

$$N_{DHN} \equiv 55.5B = n_h (K (\tau_c^h/\tau_c^o - 1) - 1) \quad (5)$$

$$K = (e^2qQ_h/h)^2 / (e^2qQ_o/h)^2$$

where B is the slope of the plot between T_1^o/T_1 and the molarity of the carbohydrate, m. The subscripts h and o represent the hydration water and pure, i.e. bulk water, respectively, and n_h represents the hydration number. Both values of (e^2qQ_h/h) and (e^2qQ_o/h) are taken as equal to that of pure water, resulting in K=1 (58). N_{DHN}, or dynamic hydration number (58), is, as defined in eq. (5), the product of the hydration number and the mobility of water molecules. We reported data of N_{DHN} which revealed the high hydration ability of trehalose relative to several gluco-oligosaccharides (59). As shown in Table 5, N_{DHN} lies in the order maltopentaose > trehalose > maltotriose > maltose. In addition, what is more important is that the magnitude of τ_c^h/τ_c^o being a measure of water molecular mobility in the neighborhood of the dissolved carbohydrate is larger for trehalose than for any other gluco-oligosaccharides studied. This finding suggests that trehalose is the most effective to induce decreased mobility of its surrounding water molecules. Taken together, this disaccharide has the characteristic hydration ability in terms of not only its large hydration number but also the remarkably lowered dynamics of its hydration water.

It has been observed that thermodynamic properties of water like partial molar heat capacity and volume, relative to the structure of aqueous solutions, show a considerable increase in the presence of trehalose (42). These values are higher in magnitude compared with those for several other mono- and disaccharides as well as polyols (42,60). It has also been reported that trehalose has a larger hydrated volume compared with other sugars (61). Combining these results with the above ^{17}O NMR data (59), it is inferred that trehalose could form stronger and more extensive hydrogen bonding with water molecules.

The information from the above experimental data is limited to water dynamics and structure averaged over an inhomogeneous sugar surface in various conformational states, and they do not provide any detailed picture about the origin of water dynamics variations depending on the stereochemistry of carbohydrates (Table 5). To address this issue, molecular dynamics (MD) simulations have been applied to solvated sugar systems. Before discussing those results, however, our attention should be for a while turned to the conformational property of trehalose itself. Molecular mechanics and quantum chemical calculations by French's group indicated that trehalose has only a single energy minimum around the glycosidic bond (62,63): the minimum is located at the glycosidic dihedaral angles of $(\phi, \varphi)=(-60°, -60°)$. Figure 8A shows the population density map for the dihedral angle distributions obtained from a MD simulation for trehalose in aqueous solution. As expected, the (ϕ, φ) angles are located only around $(-60°, -60°)$, corresponding to the gauche conformation. Consequently, this sugar has a single conformation around the glycosidic bond and its overall shape is clam shell-like (Figure 1). Such conformational rigidity of trehalose comes from the alpha,alpha-1,1 type of glycosidic linkage, which is unique to this sugar among naturally occurring gluco-disaccharides. Indeed, neotrehalose, which has an alpha,beta-1,1 configuration, has more than two stable conformations around the glycosidic linkage (Figure 8B) (62). Similarly, other types of glycosidic linkage, including (1-4), (1-6) bonds and so on, allow for multiple conformers. A recent experimental study on the interaction of trehalose with lipid membrane indicated that unlike other naturally occurring disaccharides, the two glucopyranose rings of trehalose can fit the bilayer surface and hydrogen-bond with the head groups of the phosphoholipid molecules (64). Taken together, the less flexible alpha,alpha-1,1-glycosidic linkage could be an important clue to explain the biological functions of trehalose.

To date, the MD simulation for aqueous

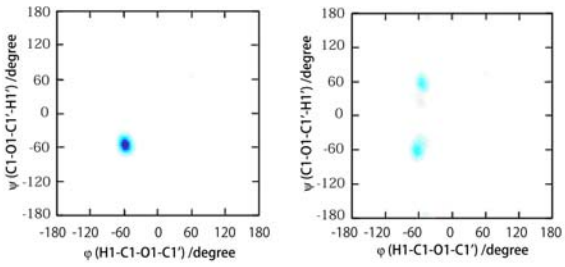

Figure 8. Population density map for the dihedral angle distributions obtained from MD simulations for trehalose (A) and neotrehalose (B) in aqueous solution.

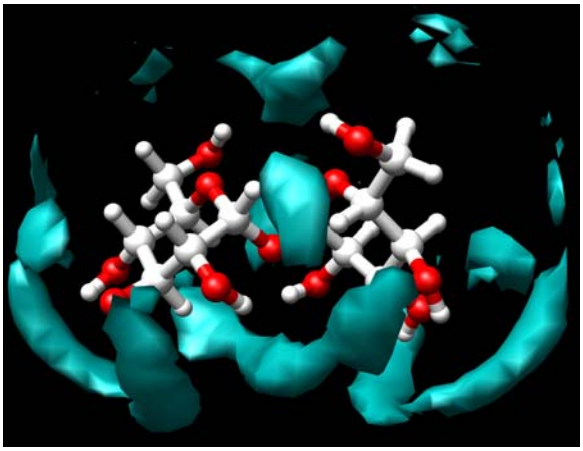

Figure 9. Distribution of water molecules around trehalose. Cloud-like regions represent iso-probability surface of water oxygen atoms.

trehalose has been reported by several groups (50,51,54,65-69). Our early MD study indicated that trehalose can hydrogen-bond with the surrounding water more extensively than maltose, leading to more restrained translational diffusion of water molecules around trehalose (65). A recent remarkable increase in computer performance allows for more rapid and accurate MD calculations for various carbohydrates in solution. Most recently, Choi *et al.* have performed systematic computational work for a series of disaccharides to obtain an atomic-level insight of unique biochemical role of the alpha,alpha-1,1-linked glucopyranoside dimer over the other glycosidically linked sugars (69). In that study, the following 13 different homodisaccharides with different glycosidic linkages were examined: 1-11 (Table 2), alpha-D-Galp-1,1-alpha-D-Galp, and alpha-D-Manp-1,1-alpha-D-Manp. Analyses of the hydration number and radial distribution function of solvent water molecules showed that there was very little hydration adjacent to the glycosidic oxygen of trehalose and that the dynamic conformation of trehalose was less flexible than any of the other sugars (Figure 8). Due to such properties, a highly anisotropic hydration shell is formed around this sugar in aqueous solution (Figure 9). In addition, they evaluated the number of long-lived hydrogen bonds defined as having a lifetime longer than 20 ps. As a result, trehalose was shown to make an average of 2.8 of long-lived hydrogen bonds with water, which is a much larger number than the average number of hydrogen bonds for the other 12 sugars. The stable hydrogen-bond network was thought to be derived from the formation of long-lived water bridges at the expense of decreasing the dynamics of the water molecules. This dynamic reduction of water by trehalose was also confirmed from data for the lowest translational diffusion coefficients and the lowest intermolecular coulombic energy of the water molecules around trehalose. These results rigorously support our ^{17}O NMR results (59). According to Choi *et al.*, trehalose is a 'dynamic reducer' for solvent water molecules, which comes from its anisotropic hydration and conformational rigidity (69). It should be again stressed that such a peculiar property of trehalose originates from the presence of its alpha,alpha-1,1-linkage.

Taken together with sections 4 and 5, trehalose is a water structure maker in the sense that it forms a highly anisotropic and unmobilized hydration shell around itself. However, this simultaneously means that the tetrahedral hydrogen bond network in water is highly perturbed by the addition of trehalose, as evidenced from the data of the isentropic partial molar compressibility $K°_{S,2}$. In this sense, trehalose is a water structure breaker as well. Such a dual character is the key to understand various biological roles of this sugar.

6. PROTEIN STABILIZATION BY TREHALOSE

Protein stabilization by trehalose in aqueous solution (15-18) is an example of the biological functions of trehalose which come from its character as a water structure maker. Timasheff and coworkers investigated the stabilization of RNase A by trehalose based on the so-called preferential hydration model (17), which insists that sugars and polyols stabilize the folded structure of proteins in solution as a result of greater preferential hydration of the unfolded state compared with the native state. Wyman linkage analysis (eq. 6) and the data of the transfer free energy on unfolding suggested that at room temperature trehalose stabilizes this protein through the preferential hydration mechanism, although at higher temperatures it tends to preferentially bind to the protein. In general, however, different proteins are expected to interact with cosolvent molecules in varied ways depending on their physicochemical properties. Indeed, trehalose has been observed to provide protection to different proteins to various extents and the efficacy of protection depends on the nature of the protein (15,70). Thus, to understand the mechanism of trehalose-mediated thermal stability of proteins in detail, Kaushik and Bhat have conducted a thorough investigation of its effect on the thermal stability in aqueous solutions of five well characterized proteins differing in their various physico-chemical properties: ribonuclease A, lysozyme, cytochrome *c*, alpha-chymotrypsinogen, and trypsin-inhibitor (18). They also used the Wyman linkage analysis (eq. (6)) to determine the relative preferential interaction of trehalose with the two end states of the proteins,

$$d(\ln K)/d(\ln a) = (n_D - n_N) = \Delta n \quad (6)$$

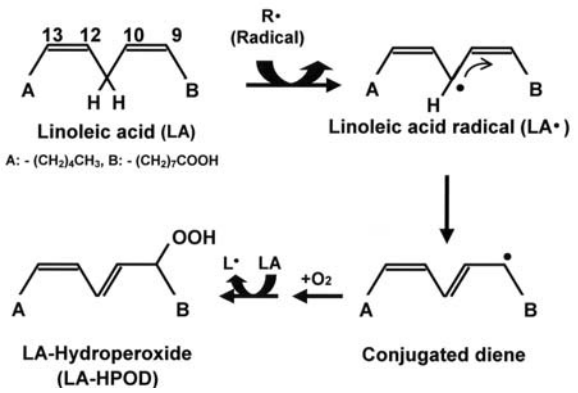

Figure 10. Peroxidation of linoleic acid.

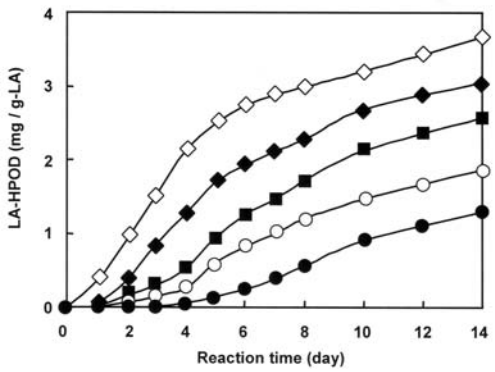

Figure 11. Inhibitive effect of trehalose on formation of linoleic acid hydroperoxide (LA-HPOD) by autoxidation. Autoxidation of linoleic acid with trehalose was carried out at 37 °C. The formation of LA-HPOD was evaluated from the observation of absorbance at 233 nm. ◊: no addition, ♦:1.46 mM, trehalose, ■: 7.30 mM trehalose, ○: 14.6 mM trehalose, ●: 29.2 mM trehalose.

where K is the equilibrium constant for the conversion reaction $N \rightarrow D$, a is the cosolvent activity, and n_D and n_N are the numbers of cosolvent molecules bound to the denatured and the native protein molecules, respectively. The Δn values obtained for the above proteins at 1.5 M trehalose vary from -7 to -4. The negative values indicate the preferential exclusion of trehalose (equivalent to preferential hydration) from the hydration shell of the protein upon denaturation.

Preferential hydration should occur when the interaction of a given cosolvent with water is stronger than its interaction with a protein. In other words, such a cosolvent is a water structure maker whose hydration shell is strongly structured. In this regard, trehalose is a good cosolvent causing preferential hydration. The preferential hydration effect should lead to a loss in the entropy of solvation upon protein denaturation, rendering the unfolded state even more unstable, and resulting in a shift of the equilibrium in favor of the native state. In addition, the water structure maker causes a significant increase of the surface tension of water. For example, at identical concentrations, trehalose increases the surface tension of water by much larger amounts compared with other sugars and polyols (18). Protein denaturation in such a solution would need additional energy to accommodate its increased surface area. Thus, the effect of surface tension is also an important factor contributing to the stabilization of a protein.

According to the preferential hydration model as described above, trehalose is expected to promote the aggregation of unfolded proteins because the aggregated state should have a smaller protein-solvent interface than their isolatedly dissolved state. However, in contradiction to this expectation, trehalose has been shown to suppress aggregation of unfolded proteins both *in vivo* (20,71) and *in vitro* (21) states. Although the underlying mechanism for such phenomena is far from being fully understood at present, a key to solve this issue may exist in another peculiar property of this sugar, that is, a specific interaction with hydrophobic compounds as described in the next section.

7. ANTIOXYDANT FUNCTION OF TREHALOSE

In addition to the protective function against water stresses, there is growing evidence that trehalose is capable of protecting proteins and unsaturated fatty acid (UFA) against oxidative damage (8). An *in vivo* study by Benaroudj *et al.* indicated that trehalose protects yeast cells and cellular proteins from damage by oxygen radicals (7). The antioxidant function on UFA has been extensively studied by our group from both experimental and theoretical viewpoints as described below (8,72,73).

As shown in Figure 10, the autoxidation of UFA is initialized by the reaction in which activated oxygen or free radicals attract hydrogen atoms from the allyl group of UFA. In the subsequent reaction, the resultant conjugated diene reacts with O_2 and another UFA molecule to produce hydroperoxy-fatty acid, followed by degradation or polymerization by the secondary oxidation reaction. The formation of hydroperoxide (HPOD) can be evaluated by monitoring the absorption (233 nm) of the conjugated diene. The effect of trehalose on HPOD formation of linoleic acid (LA) is shown in Figure 11 (73), and indicates that the amount of HPOD produced decreases depending on trehalose concentration and thus that trehalose depresses the reaction rate of the autoxidation step. However, such a phenomenon was not observed for the mixture of trehalose with UFA possessing trans type C=C double bonds such as eladic acid (18:2, trans) and linoeladic acid (18:1, trans). In addition, other disaccharides, such as sucrose, maltose and neotrehalose, showed a negligible effect on HPOD formation. Therefore, trehalose interacts specifically with UFA possessing a cis type C=C double bond (s) such as LA (18:2, cis), resulting in the depression of the formation of HPOD. Stoichiometry of this complex formation was elucidated by observing the 1H-T1 values of olefin and the adjacent methylene protons of LA. As shown in Figure 12 (8), the 1H-T1 vs. trehalose concentration curve reaches a plateau at a 2:1 molar ratio of trehalose to LA. When using oleic acid (18:1, *cis*) or alpha-linolenic acid (18:3, *cis*) as

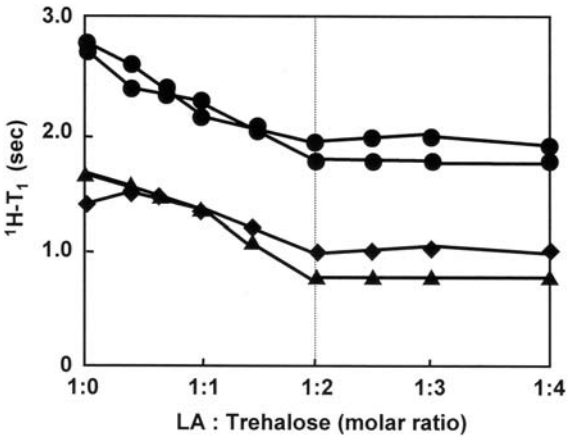

Figure 12. Effect of the molar ratio of trehalose to linoleic acid on the relaxation time of the olefin (●) and the adjacent methylene protons (▲and ♦).

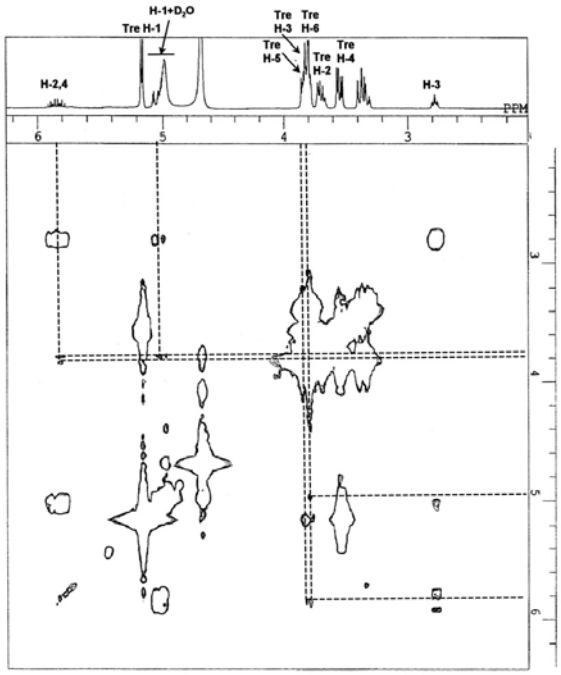

Figure 13. ^{1}H- ^{1}H NOESY spectrum of a mixture of trehalose and pentadiene (molar ratio = 2:1).

UFA, the curve reached a plateau at a molar ratio of 1:1 and 3:1, respectively. From these results, it was concluded that trehalose and a *cis* type C=C double bond forms a complex with 1:1 stoichiometry. In addition, the 6,6' site and 3,3' sites of trehalose were shown to participate in the interaction from the observation of ^{1}H-^{1}H NOESY spectrum depicted in Figure 13 (72), where pentadiene was used as a model of UFA and a clear cross-peak was observed between the olefin proton signals (5.3-5.4 ppm) of pentadiene and the 6 (6')-methylene and 3 (3') proton signals (3.8-3.9 ppm) of trehalose.

A theoretical model for the trehalose-*cis* C=C bond complex is shown in Figure 14 (72), where the OH-6' of trehalose interacts with the pi-orbital at the mid position of the double bond and simultaneously the OH-3 forms the C-H···O type of hydrogen bond at a terminal of the double bond. The complex formation energy (stabilization energy) was estimated to be 23.1 and 32.5 kJ mol^{-1} from the HF/6-31G** and B3LYP/6-31G** calculations, respectively. Moreover, Oku *et al.* gave a clear answer to the problem whether the autoxidation step in Figure 10 is really suppressed as a result of the above complex formation (8,72). Actual calculations were performed for the hydrogen abstraction reaction from the activated methylene of heptadiene as follows:

$$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$$
$$\rightarrow -CH_2-CH=CH-\bullet CH-CH=CH-CH_2-$$

As a result, when trehalose is not complexed with heptadiene, the activation energies of the reaction initiated by methyl radical are 61.9 kJ mol^{-1} (UHF/6-31G**) and 38.5 kJ mol^{-1} (UB3LYP/6-31G**), while upon complexation this increases up to 157.9 kJ mol^{-1} (UHF) and 161.6 kJ mol^{-1} (UB3LYP). These results indicate that the OH···pi and CH···O multiple hydrogen bonds can strongly affect the electronic state of the diene and that the hydrogen abstraction reaction is significantly depressed by the interaction with trehalose.

As described in section 5, trehalose has a single conformation due to the rigidity of the alpha,alpha-1,1 glycosidic bond. As a result of this, once the multiple hydrogen bond (Figure 14) is formed, it is expected to have a substantial lifetime. Such stable complex formation would be impossible in the cases of other disaccharides because of free rotation around the glycosidic bond. Interestingly, the hydrogen bonding pattern of the trehalose/diene complex shown in Figure 14 is similar to that of a theoretical model for a water dimer/ethylene complex given in ref. 74, where one water molecule binds to the pi-bond at the midpoint of the double bond and the oxygen atom of the other does to the CH group through the CH···O interaction (see Figure 7b in ref. 74). In spite of such an apparent structural similarity, a C=C double bond is thought to have higher affinity with one trehalose molecule than with two water molecules because the latter case accompanies a larger entropy loss. Thus, it is possible that trehalose preferentially binds to a double bond instead of to solvent molecules.

The finding that trehalose can form intermolecular complexes with various *cis* double bonds (8,72) leads us to expect that this sugar can also interact with benzene compounds, whose double bonds are *cis* rather than *trans*. If this is true, such a peculiar interaction may be related to the suppressive effect of this sugar on the aggregation of peptide and proteins as described in the previous section. Namely, there is a possibility that this sugar binds to aromatic side chains that are exposed to the aqueous phase upon unfolding and consequently act as a spacer to inhibit the direct contact between unfolded protein molecules. This interesting issue is now under investigation in our laboratory.

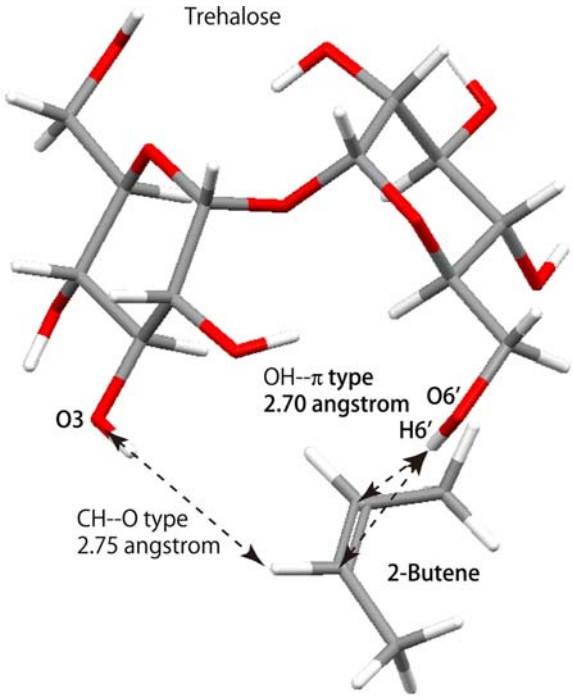

Figure 14. The optimized structures of trehalose/2-butene complex obtained from the HF/6-31G** calculation.

8. PERSPECTIVE

The uniqueness of trehalose comes from the presence of an alpha,alpha-1,1-linkage, which brings about the rigid conformation with a clam, shell-like shape. Because of its conformational rigidity, trehalose has a unique hydration characteristic: a spatially anisotropic but dynamically stable hydration shell. This in turn brings about several characteristic thermodynamic properties for its aqueous solution: relatively large amount of unfrozen water (U_w), more negative isentropic partial molar compressibility ($K°_{S,2}$), relatively large values of partial molar heat capacity and volume and larger surface tension. These results reflect the dual character of this sugar as a good water structure maker and breaker. The character as a water structure breaker explains the fact that this sugar acts as a better cryoprotectant than other disaccharides. The larger U_w value is related to a higher Tg (Figure 4), although the molecular origin of such a relation remains unclear at present. The characteristic glassy property with not only high Tg but also high ΔE_{rel} makes trehalose a superior desiccation protectant than other saccharides. The character as a water structure maker contributes to the stabilizing function on proteins in solution. Finally, the conformational rigidity leads to the peculiar interaction with unsaturated compounds with *cis* type C=C double bond (s) and thus this sugar acts as a protectant against oxidation stress. The unified view obtained here is a significant advance in understanding the limitation and further possibility of this sugar in various applications and in undertaking the molecular design of more effective protectants in the future.

9. ACKNOWLEDGMENTS

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Send correspondence to: Minoru SakuraiI, Center for Biological Resources and Informatics, Tokyo Institute of Technology, B-62 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan, Tel:81-45-924-5795, Fax: 81-45-924-5795, E-mail: msakurai@bio.titech.ac.jp

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