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RESEARCH PAPER

Effect of Sugars on the Molecular Motion of Freeze-Dried Protein Formulations Reflected by NMR Relaxation Times

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ABSTRACT

Purpose To relate NMR relaxation times to instability-related molecular motions of freeze-dried protein formulations and to examine the effect of sugars on these motions.

Methods Rotating-frame spin-lattice relaxation time ($T_{1\rho}$) was determined for both protein and sugar carbons in freeze-dried lysozyme-sugar (trehalose, sucrose and isomaltose) formulations using solid-state ^{13}C NMR.

Results The temperature dependence of $T_{1\rho}$ for the lysozyme carbonyl carbons in lysozyme with and without sugars was describable with a model that includes two different types of molecular motion with different correlation times (τ_c) for the carbon with each τ_c showing Arrhenius temperature dependence. Both relaxation modes have much smaller relaxation time constant (τ_c) and temperature coefficient (Ea) than structural relaxation and may be classified as β-relaxation and γ-relaxation. The τ_c and Ea for γ-relaxation were not affected by sugars, but those for β-relaxation were increased by sucrose, changed little by trehalose, and decreased by isomaltose, suggesting that the β-mobility of the lysozyme carbonyl carbons is decreased by sucrose and increased by isomaltose.

Conclusion $T_{1\rho}$ determined for the lysozyme carbonyl carbons can reflect the effect of sugars on molecular mobility in lysozyme. However, interpretation of relaxation time data is complex and may demand data over an extended temperature range.

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INTRODUCTION

Many researchers have recognized that the instability of pharmaceutical formulations in the amorphous state is correlated with the molecular dynamics (1–9). Correlations between instability and structural relaxation (10–18) or molecular motions with shorter timescales (β-relaxation or fast dynamics) (19–24) have been demonstrated for various amorphous formulations. For stability prediction and stabilization of freeze-dried protein (and labile small molecule) formulations, it is important to identify the motion most relevant to the instability, which largely depends on the degradation mechanisms and formulation components.

Various techniques have been used to determine molecular motions in amorphous formulations for proteins and small molecules. Structural relaxation has been characterized by dynamic mechanical measurements (25–27), isothermal microcalorimetry (28), differential scanning calorimetry (29), dielectric relaxation spectroscopy (30–32) and thermally stimulated depolarization current spectroscopy (33). Some of these techniques have also been used to characterize molecular dynamics on timescales shorter than structural relaxation. Fast dynamics of amorphous formulations on timescales much shorter than structural relaxation have been studied by neutron scattering and NMR relaxation measurements. Neutron scattering can probe atomic motions on the timescale of nanoseconds or shorter in freeze-dried formulations (22,23,34). NMR can detect atomic motions on the timescale of MHz and kHz, which are reflected by spin-lattice relaxation times in the

laboratory and rotating frames (T_1 and $T_{1\rho}$), respectively (35–37).

Although one might intuitively expect that instability of formulations would correlate best with structural relaxation because of the similar timescales of degradation and structural relaxation, correlations are often better with fast dynamics (20,22–24). For example, it has been demonstrated that NMR relaxation times T_1 and $T_{1\rho}$ are coupled with the instability of freeze-dried protein formulations. The $T_{1\rho}$ of the carbonyl carbons of freeze-dried insulin was increased by the addition of trehalose, which increased the storage stability of insulin (20). Sucrose, which increased the $T_{1\rho}$ of the protein carbonyl carbons more intensely than trehalose and stachyose, stabilized freeze-dried β-galactosidase more effectively than the others (24). These findings qualitatively suggest couplings between NMR fast dynamics and instability of freeze-dried protein formulations, but the mechanism of coupling is still unclear.

The purpose of this study is to quantify the timescales of molecular motions that are potentially coupled with instability and to elucidate the effect of sugars on these molecular motions. Here, $T_{1\rho}$ was determined for both protein and sugar carbons in freeze-dried protein formulations as a function of temperature using solid-state ^{13}C NMR. $T_{1\rho}$ is a NMR relaxation time, which does not directly indicate molecular mobility. Thus, the correlation time (τ_c) of the carbon, which indicates the time required for the carbon to rotate one radian, was determined from the temperature dependence of the observed $T_{1\rho}$. Trehalose, sucrose and isomaltose were used as excipients, and the well-studied protein lysozyme was used. Trehalose and sucrose are known to stabilize many freeze-dried proteins (20,24). Isomaltose is also a dissacharide with a similar molecular structure to trehalose and sucrose and has a T_g value between those of trehalose and sucrose. The effect of sugars on the τ_c of the carbon is discussed in relation to the stabilizing effects of these sugars. Furthermore, the temperature coefficient (apparent activation energy, Ea) of molecular motions is determined from the temperature dependence of τ_c and compared with the values reported for Ea as determined by other relaxation techniques, such as dynamic mechanical measurements, isothermal microcalorimetry and dielectric relaxation spectroscopy.

MATERIALS AND METHODS

Preparation of Freeze-Dried Formulations

Sucrose (S-9378, Sigma Chemical Co., St. Louis, MO, USA), trehalose (Pfanstiehl, Waukegan, IL, USA), isomaltose (400480–2 Seikagaku baio bijinesu Co., Tokyo, Japan) and ^{13}C-methyl isomaltose were freeze-dried with or without lysozyme (Sigma Chemical Co., St. Louis, MO, USA). ^{13}C-methyl isomaltose was prepared by methylating isomaltose with ^{13}C-methyl iodide (99% ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, MA) using dimsyl sodium as a proton removal reagent (38).

Protein solutions (50 mg/mL) were prepared after dialysis against water. Protein : sugar solutions were prepared in a one-to-one ratio by diluting the protein solution to 25 mg/mL. Samples were prepared in 5 ml tubing glass vials (1 mL fill volume) and freeze-dried in a FTS Durastop freeze-drier (FTS Kinetics, Stoneridge, NY). The shelf temperature during primary drying was set at −25°C and increased at 0.1°C/min to 40°C for secondary drying and held for 6 h. Chamber pressure throughout drying was set at 80mTorr, and in all cases product temperature was maintained below collapse temperature. Vials were sealed in the chamber under vacuum using Daikyo Florotec stoppers and stored at −20°C until use. The water content determined by the Karl Fisher method was less than 0.2%. The glass transition temperature (T_g) measured by differential scanning calorimetry (TA Instruments) was 73°C for sucrose, 114°C for trehalose, 101°C for isomaltose, 88°C for lysozyme-sucrose, 131°C for lysozyme-trehalose and 109°C for lysozyme-isomaltose.

Determination of $T_{1\rho}$ of Lysozyme Carbonyl and Sugar Carbons by ^{13}C Solid-State NMR

The freeze-dried sample was added to a 4 mm Zirconia MAS rotor with a Macor cap (Wilmad LabGlass, Vineland, NJ), firmly, with the packing tool in a glove bag purged with dry nitrogen (relative humidity < 2%).

The $T_{1\rho}$ of each lysozyme carbonyl carbon and sugar carbon was determined at nominal (set) temperatures ranging from −70°C to 150°C (actual sample temperature: −57.3°C to 130.7°C) using a ^{13}C CP/MAS NMR operating at a proton resonance frequency of 300 MHz (Bruker DMX 300). The software used was Xwinnmr 2.6. Spin-locking field was equivalent to 62.5 kHz (^1H 90° pulse length was 4.0 μs). The spinning speed was 10 kHz. The contact time was 1.0 ms, and the recycling delay was 5 s. The maximum length of the spin locking pulse was varied with the $T_{1\rho}$ value from 4 ms to 30 ms. Signals were obtained at five spin locking pulse lengths. Signal acquisition was performed for 4–5 h at each data point except for the measurements to examine changes in $T_{1\rho}$ during heating below the T_g. Temperature was calibrated using ^{207}Pb MAS spectra of solid lead nitrate, as previously reported (39).

The NMR spectrum obtained for freeze-dried lysozyme is shown in Fig. 1, and those for trehalose, sucrose,

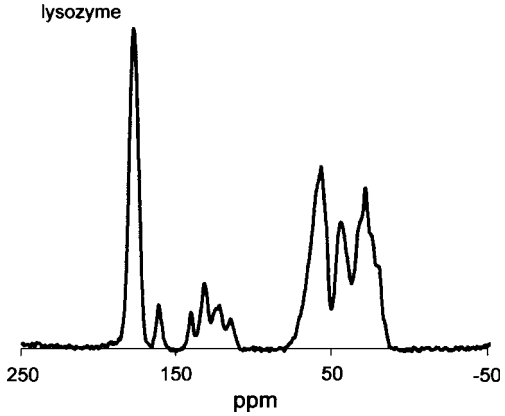

Fig. 1 NMR spectrum of freeze-dried lysozyme.

isomaltose, lysozyme-trehalose, lysozyme-sucrose and lysozyme-isomaltose are shown in Fig. 2. For the measurement of $T_{1\rho}$ for the lysozyme carbonyl carbons, the peak at approximately 175 ppm was used (observed $T_{1\rho}$ represents the average of that for all carbonyl carbons in all molecules of lysozyme). For the $T_{1\rho}$ measurement of trehalose carbon, the peak at 92 ppm belonging to the methine carbons (C-1 and C-1′) was used. The peak at 104 ppm belonging to the carbon (C-1) was used for the $T_{1\rho}$ measurement of sucrose carbon (40). For the $T_{1\rho}$ of isomaltose comprised of two anomers, the peak at 97 ppm, which mainly belongs to the methine carbon (C-1), was used. $T_{1\rho}$ was calculated by fitting the signal decay to a mono-exponential equation. τ_c was calculated from the $T_{1\rho}$ using the parameters estimated by fitting the $T_{1\rho}$ data to an equation describing the relationship between $T_{1\rho}$ and τ_c (described in the Discussion section) with the Origin 8.1 software (OriginLab Co., Northampton, MA).

Sub-T_g Heating of Freeze-Dried Lysozyme-Trehalose and Lysozyme-Sucrose

The effect of sub-T_g heating (i.e., annealing) on the $T_{1\rho}$ of the lysozyme carbonyl carbons was examined using freeze-dried lysozyme-trehalose and lysozyme-sucrose. Freeze-dried samples packed in the rotor were heated at 62°C for 8 h in the NMR probe with spinning at 10 kHz. Then, signal acquisition was started after temperature was lowered or raised to the target temperature.

In addition, the time dependence of changes in $T_{1\rho}$ during sub-T_g heating was examined by acquiring signals at −40°C, and then at 62°C, as a function of time. The duration required for a temperature change from −40°C to 62°C was 0.5 h. Signal acquisition at 62°C was started immediately after the temperature became constant and at intervals thereafter. Signal acquisition was carried out for 3 h at each time point.

RESULTS

Temperature Dependence for $T_{1\rho}$ of Lysozyme Carbonyl Carbons

The temperature dependence of the relaxation time is critical for the evaluation of the fundamental time constant, the molecular correlation time (τ_c). The time course of $T_{1\rho}$ relaxation for the carbonyl carbons of lysozyme was describable with the mono-exponential equation for all samples, both in the absence and the presence of sugars. Figure 3 shows the temperature dependence of the $T_{1\rho}$ calculated from the mono-exponential time course. The $T_{1\rho}$ of the carbonyl carbons in the absence of sugars and in the presence of trehalose and isomaltose exhibited a complex temperature dependence that shows two minima in the temperature ranges above 50°C and below 10°C, as well as a maximum at a temperature between 10°C and 90°C. For lysozyme freeze-dried with sucrose, $T_{1\rho}$ was not determined at temperatures above 90°C because of the possible complicating effects of a glass transition.

For the $T_{1\rho}$ minimum in the lower temperature range, the addition of sugars did not change the temperature at which the $T_{1\rho}$ minimum occurred. In contrast, for the $T_{1\rho}$ minimum in the higher temperature range, the minimum was impacted by the sugars with the effect of sugars on the temperature of $T_{1\rho}$ minimum varying with the sugar. Isomaltose significantly shifted the temperature of the $T_{1\rho}$ minimum to a lower temperature, whereas the effect of trehalose was not significant. Although the $T_{1\rho}$ minimum in the higher temperature range could not be directly observed for sucrose, sucrose shifted the temperature of the $T_{1\rho}$ maximum (which occurs between the two minima) to a higher temperature, suggesting that the $T_{1\rho}$ minimum is sifted to a higher temperature. Note also that the temperature of the $T_{1\rho}$ maximum was shifted to a lower temperature by isomaltose, whereas the effect of trehalose was not significant.

The value of $T_{1\rho}$ at the $T_{1\rho}$ minimum was increased by trehalose and isomaltose for both $T_{1\rho}$ minima in the lower and higher temperature ranges. Sucrose also increased the value of $T_{1\rho}$ at the $T_{1\rho}$ minimum in the lower temperature range. Trehalose exhibited the greatest effect on the $T_{1\rho}$ value at the minimum.

Effect of Sub-T_g Heating on $T_{1\rho}$ of Lysozyme Carbonyl Carbons

Annealing of freeze-dried formulations at temperatures below and near the T_g is well known to increase the α-relaxation time of the formulation (41). Thus, the change in $T_{1\rho}$ with time upon heating at a temperature below T_g was determined for the lysozyme carbonyl carbons in freeze-

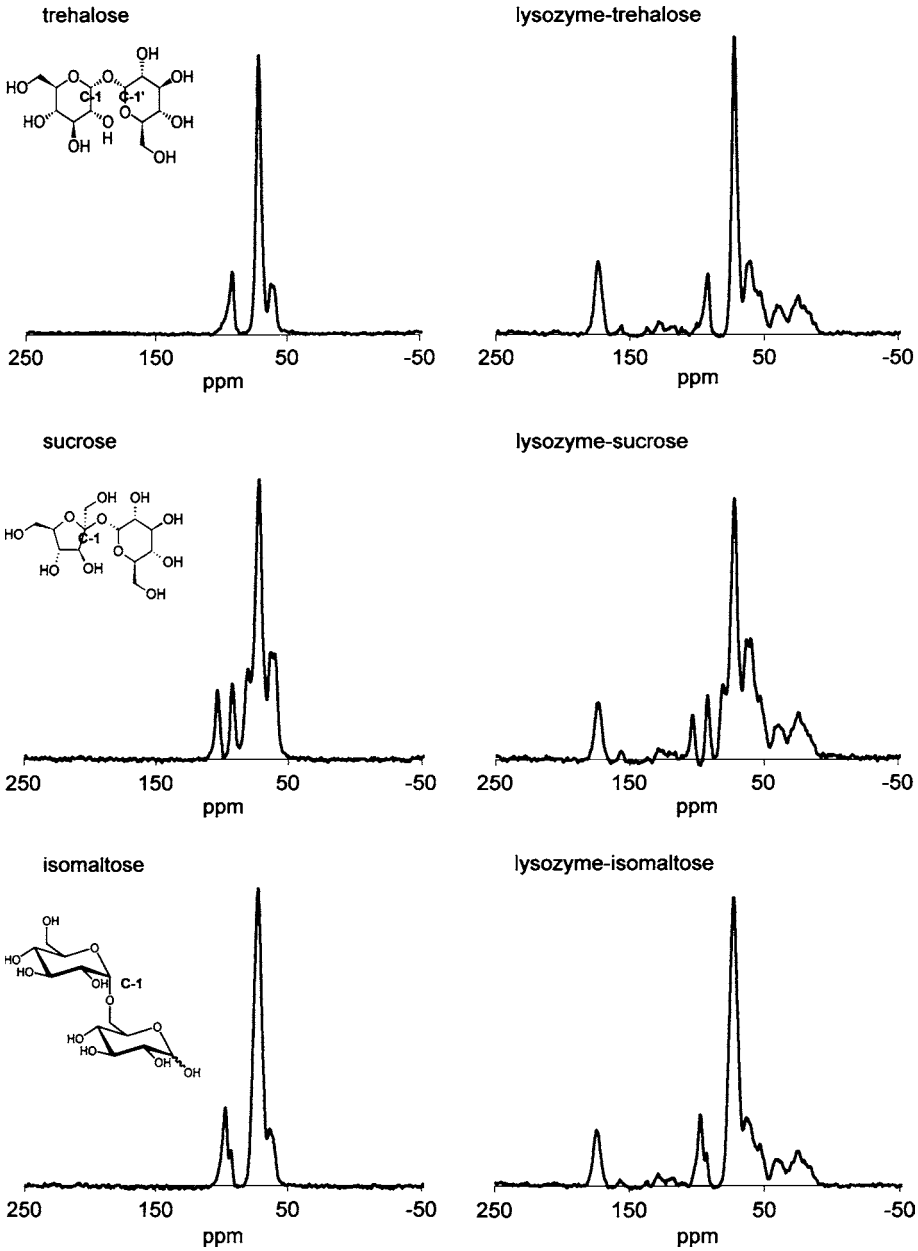

Fig. 2 NMR spectra of freeze-dried sugars and freeze-dried lysozyme with sugars.

dried lysozyme-trehalose and lysozyme-sucrose. Figure 4 shows the time dependence of changes in the $T_{1\rho}$ of the lysozyme carbonyl carbons associated with a temperature change from −40°C to 62°C. Similar changes were observed for both freeze-dried lysozyme-trehalose (Fig. 4a) and lysozyme-sucrose (Fig. 4b). Immediately after temperature was raised from −40°C to 62°C, $T_{1\rho}$ increased to a value similar to that determined at 62.3°C without the heating-cooling sequence, as shown in Fig. 3. Then, at a time shortly (≈ 3 hr) after the temperature rise, $T_{1\rho}$ sharply decreased. Thereafter, $T_{1\rho}$ gradually decreased further to an apparent equilibrium value.

Figure 5 compares the temperature dependence of $T_{1\rho}$ before and after heating at 62°C for 8 h. For both freeze-dried lysozyme-trehalose and lysozyme-sucrose, the values of $T_{1\rho}$ at temperatures above 0°C were greatly decreased by the heating treatment, whereas only small changes were observed at lower temperatures. The V-shaped temperature dependence of $T_{1\rho}$ observed for the sucrose system in the lower temperature range before heating was widened and the minimum moved toward higher temperature. For the trehalose system, the $T_{1\rho}$ minimum in the lower temperature range became obscure. We also found that the minimum in the high temperature range observed before heating was eliminated. These changes in $T_{1\rho}$ caused by sub-T_g heating indicate changes in the mobility of the carbon, the details of which will be described in the Discussion section.

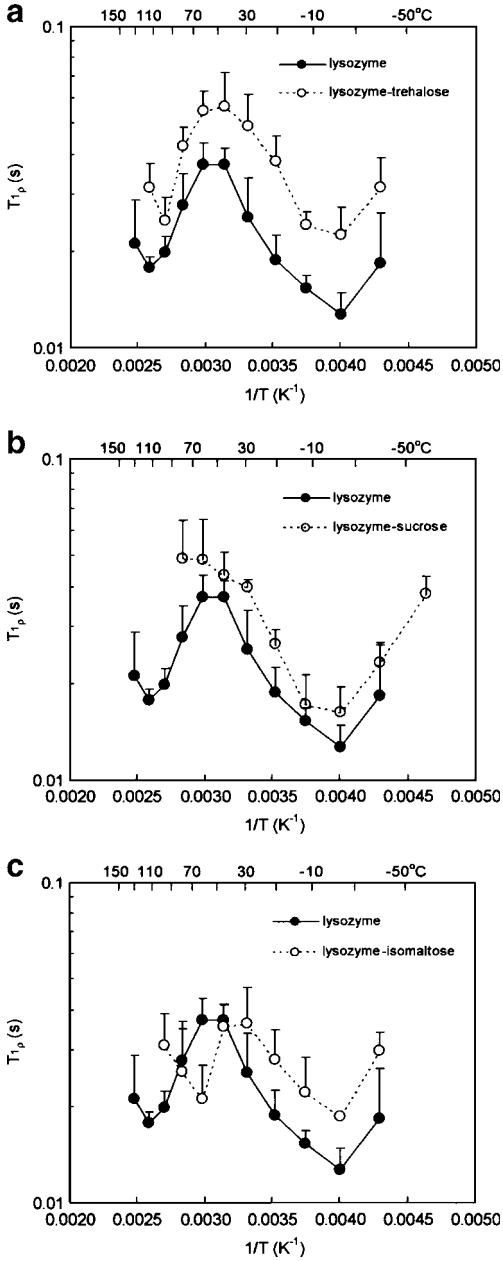

Fig. 3 Temperature dependence of $T_{1\rho}$ for lysozyme carbonyl carbons in freeze-dried lysozyme (**a**, **b**, **c**), lysozyme-trehalose (**a**), lysozyme-sucrose (**b**) and lysozyme-isomaltose (**c**) The error bars represent standard deviation ($n = 3$).

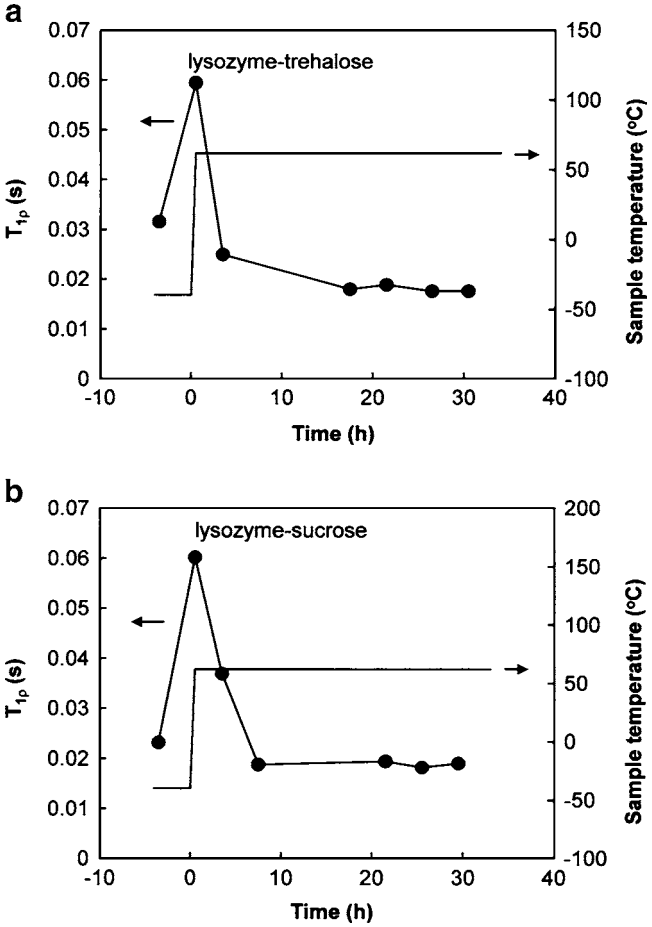

Fig. 4 Time dependence of changes in $T_{1\rho}$ of lysozyme carbonyl carbons in freeze-dried lysozyme-trehalose and lysozyme-sucrose. Temperature was increased from −40°C to 62°C at a time point of −0.5 h. The temperature reached 62°C at a time point of zero. Signal acquisition at 62°C was started at a time point of zero and at intervals thereafter. Signal acquisition was carried out for 3 h at each time point.

Temperature Dependence of $T_{1\rho}$ for Sugar carbon

The carbons of trehalose, sucrose and isomaltose (C-1 in Fig. 2) showed a peak separated from the peaks of the other methine carbons. Temperature dependence for the $T_{1\rho}$ of these carbons in the presence and absence of lysozyme is shown in Fig. 6 for the trehalose and sucrose carbons and in Fig. 7 for isomaltose. The addition of lysozyme did not bring about significant changes in the temperature dependence for the sugar carbons. The decrease in $T_{1\rho}$ observed in the high temperature range for the sucrose carbon was shifted to higher temperature by the addition of lysozyme most likely because of higher T_g of the lysozyme-sucrose system compared to the sucrose system.

Figure 7 also shows temperature dependence for the $T_{1\rho}$ of the methyl carbon introduced to the hydroxyl group of isomaltose. The methyl carbon exhibited a temperature dependence qualitatively similar to that of the methine carbon.

DISCUSSION

Mobility of Lysozyme Freeze-Dried with Sugars

The relationship between the correlation time τ_c and $T_{1\rho}$ of a given carbon can be described by Eq. 1, when

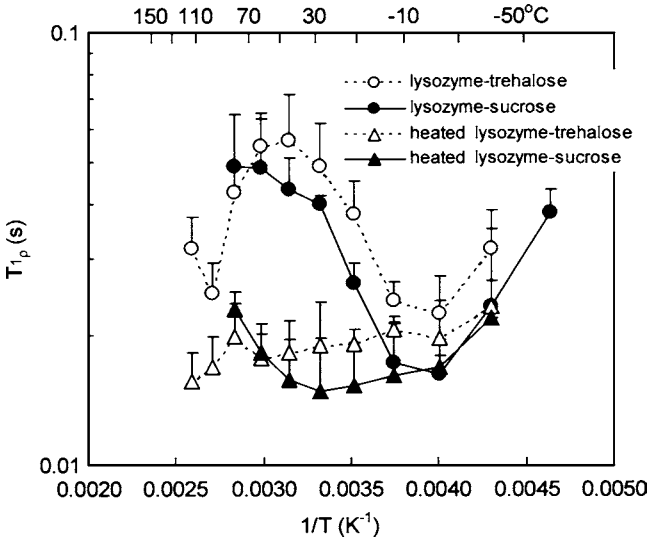

Fig. 5 Effect of heating on $T_{1\rho}$ of lysozyme carbonyl carbons in freeze-dried lysozyme-trehalose and lysozyme-sucrose. Heating was carried out at 62°C for 8 h. The error bars represent standard deviation ($n = 3$).

the carbon has a single type of motion that shows a single τ_c.

$$\frac{1}{T_{1\rho}} = \frac{A\tau_c}{1 + 4\omega_1^2\tau_c^2} \tag{1}$$

where ω_1 is the strength of spin-locking and A is a constant determined by the gyromagnetic ratio of carbon, the number of protons involved in proton-carbon dipole interaction, which causes spin-lattice relaxation, and the distance between the carbon and the proton involved in the interaction. Because $T_{1\rho}$ becomes a minimum when $\tau_c = 1/2\omega_1$, the value of A can be determined from the

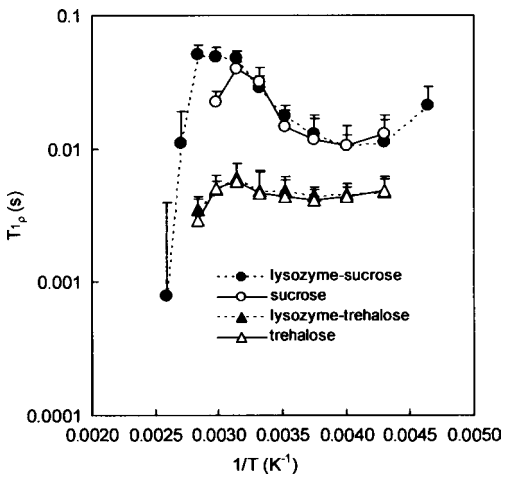

Fig. 6 Temperature dependence for $T_{1\rho}$ of carbons C-1 of trehalose and sucrose with and without lysozyme. The error bars represent standard deviation ($n = 3$).

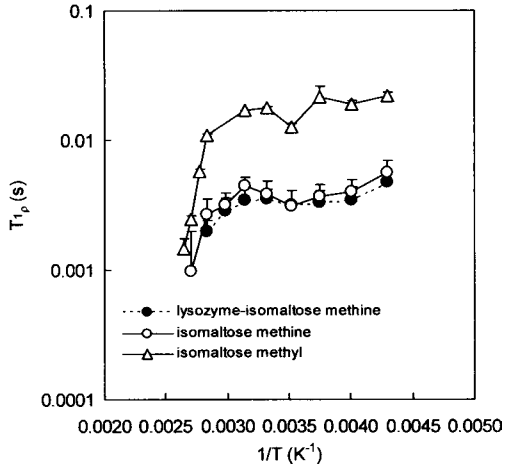

Fig. 7 Temperature dependence for $T_{1\rho}$ of carbon C-1 of isomaltose with and without lysozyme and temperature dependence for $T_{1\rho}$ of the methyl carbon of methylated isomaltose. The error bars represent standard deviation ($n = 3$).

$T_{1\rho}$ value observed at the minimum ($T_{1\rho}$(min)) according to Eq. 2.

$$A = \frac{4\omega_1}{T_{1\rho}(\min)} \tag{2}$$

When the carbon has multiple types of motion with different τ_c values, $T_{1\rho}$ is described by an equation that sums the term for each τ_c value (Eq. 3).

$$\frac{1}{T_{1\rho}} = \sum_{i=1}^{n} \frac{A_i\tau_{c,i}}{1 + 4\omega_1^2\tau_{c,i}^2} \tag{3}$$

The analysis of NMR relaxation times using an equation comprising multiple terms of τ_c has been reported for the T_1 of ^2H of $[Co(H_2O)_6][SiF_6]$ (42) and the T_1 and $T_{1\rho}$ of ^{19}F for flufenamic acid dispersed in PVP (Aso *et al.*, unpublished data). The same approach can be applied to the $T_{1\rho}$ of the lysozyme carbonyl carbons (Fig. 3) by assuming that the carbon has two different types of molecular motion with different τ_c values and that each τ_c shows an Arrhenius type of temperature dependence (Eq. 4).

$$\tau_c = \tau_0 \exp\left(\frac{E_a}{RT}\right) \tag{4}$$

The regression curve obtained for each molecular motion is shown in Fig. 8a, and the temperature dependence of τ_c for each motion is compared in Fig. 8b, which also includes the temperature dependence of α-relaxation time as calculated by the Vogel-Tammann-Fulcher (385 kJ/mol) and the Adam-Gibbs-Vogel equations (29) (123 kJ/mol) at temperatures above and below T_g, respectively. The T_g and the fragility parameter were assumed to be 130°C and 50 (16), respectively. The two types of motion observed for the

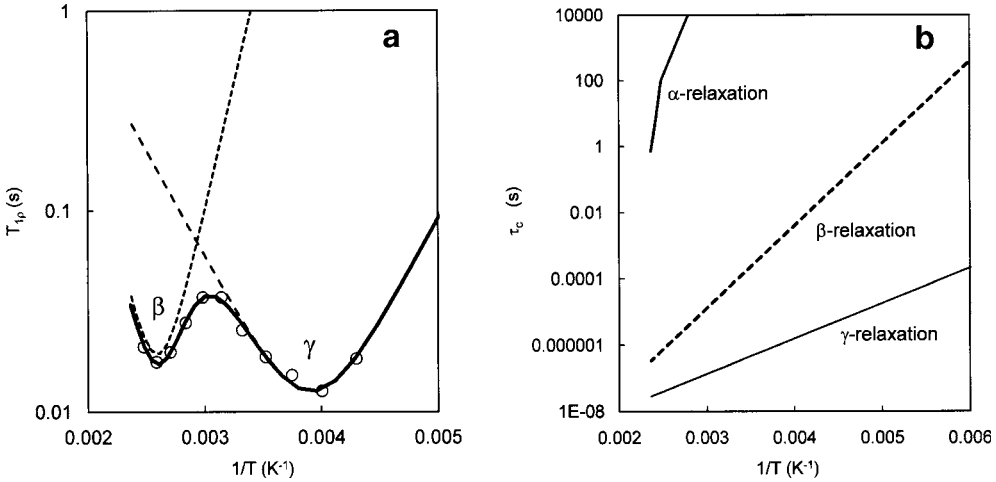

Fig. 8 (**a**) Regression curves for $T_{1\rho}$ of lysozyme carbonyl carbons in freeze-dried lysozyme obtained by assuming two different types of motion with different τ_c values. (**b**) Comparison of temperature coefficients of β- and γ-relaxation of lysozyme carbonyl carbons with that of α-relaxation time calculated assuming a T_g of 130°C and a fragility parameter of 50.

lysozyme carbonyl carbons revealed much smaller values of τ_c and Ea (i.e., slopes) than α-relaxation. The relaxation observed at higher temperatures showed a larger Ea and is referred to as β-relaxation in this paper. The other relaxation, observed at lower temperatures, is referred to as γ-relaxation. The parameters of $T_{1\rho}$(min), τ_0 and Ea were estimated to be 19 ms, 4×10^{-13} and 48 kJ/mol, respectively, for β-relaxation. For γ-relaxation, the parameters of $T_{1\rho}$ (min), τ_0 and Ea were estimated to be 13 ms, 9×10^{-11} and 20 kJ/mol, respectively.

The temperature dependence for the $T_{1\rho}$ of the lysozyme carbonyl carbons in the presence of sugars was also describable with a model that assumes that the carbon has two different types of molecular motion with different τ_c values, with each τ_c showing Arrhenius temperature dependence. The regression curves obtained for the freeze-dried lysozyme-trehalose, lysozyme-sucrose and lysozyme-isomaltose are shown in Fig. 9. The estimated parameters of $T_{1\rho}$(min) and Ea for β- and γ-relaxation are listed in Table I. The values of τ_c at 25°C calculated using these

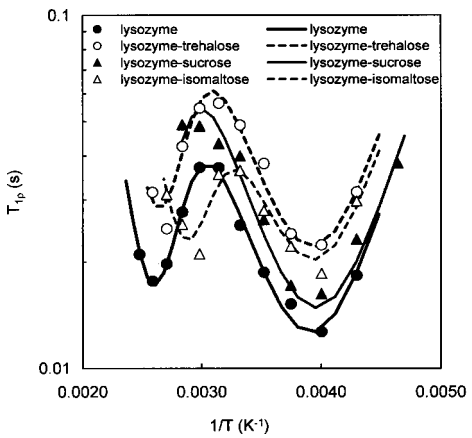

Fig. 9 Regression curves for $T_{1\rho}$ of lysozyme carbonyl carbons in lysozyme freeze-dried with sugars.

estimated parameters are also compared in Table I. The addition of sugars did not change the τ_c of the lysozyme carbonyl carbons or the Ea of τ_c for γ-relaxation. In contrast, τ_c for β-relaxation was increased by sucrose and decreased by isomaltose. Trehalose slightly decreased τ_c. The orders of τ_c and Ea for β-relaxation are as follows: sucrose > none > trehalose > isomaltose, although the standard errors in Ea make comparisons with pure lysozyme uncertain. These findings suggest that the mobility of the lysozyme carbonyl carbons is decreased by sucrose and increased by isomaltose. Because few data are available addressing the stability of freeze-dried lysozyme in the presence of sugars, the precise effect of sucrose on lysozyme stability is not known. However, the decrease in β-mobility brought about by sucrose may be related to the greater stabilizing effect of sucrose than trehalose, which was observed with some freeze-dried protein-sugar systems reported earlier (24,34).

The value of $T_{1\rho}$ minimum for the lysozyme carbonyl carbons shown in Table I varied with the sugars. The constant A in Eq. 1, which determines $T_{1\rho}$, is inversely proportional to the sixth power of the distance between the carbon and the proton that relaxes the carbon nuclei through dipole-dipole interaction (43). Thus, a small change in the distance between the carbon and proton nucleus results in a significant change in $T_{1\rho}$. The change in the estimated $T_{1\rho}$(min) caused by the addition of sugars suggests that the molecular structure of lysozyme is slightly changed by the sugars, such that the distance between the carbon and proton involved in nuclear relaxation is changed. The change in the value of $T_{1\rho}$ minimum caused by sucrose is smaller than that caused by trehalose and isomaltose, such that the change in the molecular structure of lysozyme caused by sucrose appears to be smaller than those caused by trehalose and isomaltose, which is curious in view of the fact that the apparent impact of sucrose on mobility (i.e., τ_c) is quite significant.

Table I Estimated Parameters of $T_{1\rho}$(min) and Ea for β- and γ-Relaxation, and Calculated τ_c Values at 25°C

Formulation	β -relaxation				γ-relaxation			
	τ_0 (s)	Ea (kJ/mol)	$T_{1\rho}$ (min) (ms)	τ_c (25°C) (ms)	τ_0 (s)	Ea (kJ/mol)	$T_{1\rho}$ (min) (ms)	τ_c (25°C) (μs)
lysozyme [a]	4×10^{-13} (2×10^{-12})	48 (14)	19 (4)	0.1	9×10^{-11} (2×10^{-10})	20 (4)	13 (3)	0.3
lysozyme-trehalose [b]	–	47 (1)	32 (7)	0.07	–	20 (0)	22 (2)	0.3
lysozyme-sucrose [c]	–	49 (1)	–	0.2	–	20 (0)	15 (2)	0.3
lysozyme-isomaltose [b]	–	43 (1)	28 (6)	0.02	–	20 (0)	20 (2)	0.3

() denotes standard error as provided by the non-linear regression

[a] Parameters of τ_0, Ea and $T_{1\rho}$(min) for each of the β- and γ-relaxation were evaluated by fit of the theoretical model (Eqs. 3 and 4) to the data.

[b] Parameters of Ea and $T_{1\rho}$(min) for each of the β- and γ-relaxation were estimated using the value of τ_0 obtained for lysozyme without sugars.

[c] The parameter Ea for β-relaxation was estimated using the values of τ_0 and $T_{1\rho}$(min) obtained for lysozyme without sugars, and parameters of Ea and $T_{1\rho}$(min) for γ-relaxation were estimated using the value of τ_0 obtained for lysozyme without sugars.

The value of apparent activation energy (i.e., temperature coefficient, Ea) determined for the β-relaxation of the lysozyme carbonyl carbons, shown in Table I, ranges between 43 to 50 kJ/mol, with the values for pure lysozyme and both the sucrose and trehalose systems being essentially the same but with the Ea for the isomaltose system being lower. By contrast, the Ea values for the γ-relaxation are all much smaller and essentially equal at around 20 kJ/mol. These Ea values are greater than those reported for fast dynamics of amorphous formulations determined by NMR relaxation measurements. For example, the apparent activation energy was determined to be 4.2 kJ/mol and 2.1 kJ/mol for the motion of the methine carbon in freeze-dried dextran and that of the protein carbonyl carbons in freeze-dried bovine serum γ-globulin, respectively, from NMR relaxation times (35). The dynamics of the PVP ring carbon and the sucrose methine carbon in freeze-dried PVP-sucrose mixtures, as determined by T_1 and $T_{1\rho}$, show apparent Ea values less than 10 kJ/mol. Moreover, the dynamics of the PVP ring carbon in freeze-dried PVP, determined by $T_{1\rho}$, exhibit a very small apparent Ea of 0.8 kJ/mol (36). These small apparent activation energies evaluated from the temperature dependence of NMR relaxation times, which are too small to be interpreted in terms of an activated state kinetic model, may be attributed to the invalid assumption that the atom of interest has only a single type of motion with a single τ_c value. When the atom has multiple types of motion with different τ_c values, the Ea value determined in a temperature range near $T_{1\rho}$ maximum according to this analysis has no sound theoretical foundation and may have no physical meaning as an activation energy. The analysis of NMR relaxation time according to a multiple-dynamic model, as described in this paper, is necessary to determine Ea for the individual type of motion. The value of Ea observed for freeze-dried lysozyme-sugar systems in this study is much smaller than the Ea for alpha relaxation for freeze-dried trehalose (145 kJ/mol) and freeze-dried sucrose (225 kJ/mol) as determined by isothermal microcalorimetry (28), calculated using β=0.4, but comparable to those for freeze-dried lactose by dielectric relaxation spectroscopy (72 kJ/mol and 52 kJ/mol for β- and γ-relaxation, respectively) (32).

The value of Ea determined for molecular motions in freeze-dried formulations is one of the basic clues for exploring the molecular motion most relevant to the instability of freeze-dried formulations. For example, if a certain motion shows an Ea value that is markedly different from the activation energy for degradation, it seems likely[1] that this motion may be excluded from the candidate motions responsible for the instability. The values of Ea determined for the β-relaxation of the lysozyme carbonyl carbons in this study are comparable to the values of activation energy for degradation observed for freeze-dried formulations as described below, suggesting correlations between β-relaxation and instability. Few data on activation energy have been reported for degradation in freeze-dried formulations, but Ea can be calculated from the Arrhenius plots of the apparent rate constants reported for degradation in freeze-dried formulations. For example, the Ea of the hydrolysis rate of cephalothin freeze-dried with dextran was calculated to be about 50 kJ/mol; Ea of 46 kJ/mol was calculated for acetyl transfer reaction between aspirin and sulfadiazine freeze-dried with poly(vinylpyrrolidone) (14). For degradation of freeze-dried proteins, 63 kJ/mol for aggregation of β-galactosidase freeze-dried with sugars (24), 50 kJ/mol for β-galactosidase freeze-dried with polyvinylalcohol (12), 67 kJ/mol for degradation of insulin freeze-dried with PVP (18).

[1] This statement is equivalent to stating that the motion represented by the relaxation process may have nothing directly to do with the motion required for degradation, if the "coupling coefficient" relating a relaxation time constant for a given microstate with degradation within that microstate, which has been discussed in a reference (6), is much less than unity.

Effect of Sub-T_g Heating on the Mobility of Lysozyme Carbonyl Carbons

Annealing of freeze-dried formulations at temperatures below and near the T_g is well known to increase α-relaxation time and to stabilize the formulations (41). For the freeze-dried lysozyme-trehalose and lysozyme-sucrose, the $T_{1\rho}$ value obtained immediately after the temperature was raised from −40°C to 62°C was close to the value determined at 62.3°C without heating or cooling (Fig. 3), as shown in Fig. 4. Thereafter, $T_{1\rho}$ decreased with time and became roughly constant within several hours. This finding suggests that the β- and γ-relaxations of lysozyme at the time point immediately after the temperature increase is different from those of lysozyme heated at 62°C for several hours.

As shown in Fig. 5, the $T_{1\rho}$ of the protein carbonyl carbons observed at temperatures below 0°C was not significantly affected by heating at 62°C for 8 h, but $T_{1\rho}$ above 0°C was greatly decreased by heating. The V-shaped temperature dependence of $T_{1\rho}$ for γ-relaxation was widened and moved toward a higher temperature range both for trehalose and sucrose. These findings suggest that the average τ_c for all carbonyl carbons in the lysozyme molecule is increased after heating and also that carbonyl carbons at different sites of the lysozyme molecule are affected by the heating treatment in varying degrees from each other, such that the range of τ_c for γ-relaxation of the lysozyme carbonyl carbons is widened. The effect of heating on the β-relaxation is not clear because of more limited $T_{1\rho}$ data. However, the finding that the $T_{1\rho}$ minimum for β-relaxation of the freeze-dried lysozyme-trehalose observed before heating is not observed after heating in the temperature range studied suggests that the $T_{1\rho}$ minimum for β-relaxation is shifted to a higher temperature by heating. Thus, both β- and γ-relaxations appear to be slowed by heating in a manner similar to the annealing effects on α-relaxations.

Mobility of Sugars Freeze-Dried with Lysozyme

The temperature dependence for the $T_{1\rho}$ of trehalose, sucrose and isomaltose carbons (Figs. 5 and 6) exhibits $T_{1\rho}$ minima in a similar temperature range as for the $T_{1\rho}$ of the lysozyme carbonyl carbons. This suggests that the sugar carbons have motions with similar τ_c values as the β- and γ-relaxation of the protein carbonyl carbons. Here, the values of $T_{1\rho}$ for the sucrose carbon are greater than those for the trehalose and isomaltose methine carbon. This is expected because the sucrose carbon (C-1) has no proton directly binding to the carbon, while trehalose and isomaltose methine carbons have a proton directly binding to the carbon. The rate of spin-lattice relaxation depends on the number of the protons that cause dipole-dipole interaction with the carbon, leading to spin-lattice relaxation, as well as the distance between the proton and the carbon (C-1). More protons and a shorter distance both lead to faster spin-lattice relaxation. The spin-lattice relaxation of sucrose carbon (C-1) is slower than that of trehalose and isomaltose, because the proton involved in the dipole-dipole interaction with the carbon (C-1) in the sucrose system is at a greater distance from the carbon compared to the trehalose and isomaltose systems.

The temperature dependence for the $T_{1\rho}$ of sugar carbons was not significantly changed by the addition of lysozyme. This finding means that the motions of sugar carbons with similar τ_c values as the β- and γ-relaxation of the protein carbonyl carbons are not affected by interaction with lysozyme. This finding is in contrast to the observation that the β- and γ-relaxations of the lysozyme carbonyl carbons are affected by interaction with the sugars (Table I). This difference may be explained by assuming that the sugar induces conformational changes in the protein, such that the mobility of the protein carbonyl carbons is significantly changed but mobility of the sugar carbon is changed by interaction with the protein only for the carbons involved in the interaction. Mobility of the carbons that are not involved in the interaction also contributes to the determined $T_{1\rho}$ value, such that the measured $T_{1\rho}$ for the sugar is not sensitive to the interaction with protein.

The $T_{1\rho}$ of the methyl carbon of ^{13}C-methylated isomaltose decreases rapidly as temperature increases near and above T_g (Fig. 7). This finding suggests that the motion of the methyl carbon, which is speculated to be much faster than the carbon (C-1), is coupled with structural relaxation since structural relaxation shows strong temperature dependence near T_g. This observation is in contrast to the general concept that the fast motion of side chains is generally independent of structural relaxation.

CONCLUSION

The temperature dependence for the $T_{1\rho}$ of the lysozyme carbonyl carbons in freeze-dried lysozyme with and without sugars (trehalose, sucrose and isomaltose) was describable with a model that assumes that the carbon has two different types of molecular motion with different τ_c values and that each τ_c shows an Arrhenius type of temperature dependence. A single relaxation mode is not consistent with the data, meaning that interpretation of relaxation time data is complex and may demand data over an extended temperature range. If $T_{1\rho}$ is determined in a temperature range near $T_{1\rho}$ maximum, simply observing that an increase in temperature reduces $T_{1\rho}$ does not necessarily mean that the

$T_{1\rho}$ measured is directly proportional to the correlation time, τ_c, nor is the temperature coefficient of $T_{1\rho}$ necessarily a good measure of the activation energy of a single process. Further, trends in $T_{1\rho}$ with formulation determined in this temperature range may not necessarily be predictive of trends in molecular mobility.

Lysozyme carbonyl carbons in the absence of sugars revealed two types of motion with much smaller values of τ_c and Ea than structural relaxation: a motion with a τ_c of 1×10^{-4} s at 25°C and an Ea of 48 kJ/mol (β-relaxation), and another faster motion with a τ_c of 3×10^{-7} s at 25°C and an Ea of 20 kJ/mol (γ-relaxation). Addition of sugars does impact the mobility of lysozyme, as evidenced by the impact of sugars on the values of τ_c and Ea. The τ_c and Ea for β-relaxation were increased by the addition of sucrose and decreased by the addition of trehalose and isomaltose. The orders of τ_c and Ea are as follows: sucrose > none > trehalose > isomaltose, suggesting that the β-mobility of the lysozyme carbonyl carbons is significantly decreased by sucrose but increased by isomaltose.

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