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*Research Paper*

# β-Relaxation of Insulin Molecule in Lyophilized Formulations Containing Trehalose or Dextran as a Determinant of Chemical Reactivity

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***Purpose.*** The purpose of this study was to elucidate whether the degradation rate of insulin in lyophilized formulations is determined by matrix mobility, as reflected in glass transition temperature ($T_g$), or by β-relaxation, as reflected in rotating-frame spin-lattice relaxation time $(T_{1\rho})$.
***Methods.*** The storage stability of insulin lyophilized with dextran was investigated at various relative humidities (RH; 12–60%) and temperatures (40–90°C) and was compared with previously reported data for insulin lyophilized with trehalose. Insulin degradation was monitored by reverse-phase high-performance liquid chromatography. Furthermore, the $T_{1\rho}$ of the insulin carbonyl carbon in the lyophilized insulin–dextran and insulin–trehalose systems was measured at 25°C by $^{13}C$ solid-state NMR, and the effect of trehalose and dextran on $T_{1\rho}$ was compared at various humidities.
***Results.*** The degradation rate of insulin lyophilized with dextran was not significantly affected by the $T_g$ of the matrix, even at low humidity (12% RH), in contrast to that of insulin lyophilized with trehalose. The insulin–dextran system exhibited a substantially greater degradation rate than the insulin–trehalose system at a given temperature below the $T_g$. The difference in degradation rate between the insulin–dextran and insulin–trehalose systems observed at 12% RH was eliminated at 43% RH. In addition, the $T_{1\rho}$ of the insulin carbonyl carbon at low humidity (12% RH) was prolonged by the addition of trehalose, but not by the addition of dextran. This difference was eliminated at 23% RH, at which point the solid remained in the glassy state. These findings suggest that the β-relaxation of insulin is inhibited by trehalose at low humidity, presumably as a result of insulin–trehalose interaction, and thus becomes a rate determinant. In contrast, dextran, whose ability to interact with insulin is thought to be less than that of trehalose, did not inhibit the β-relaxation of insulin, and thus, the chemical activational barrier (activation energy) rather than β-relaxation becomes the major rate determinant.
***Conclusions.*** β-Relaxation rather than matrix mobility seems to be more important in determining the stability of insulin in the glassy state in lyophilized formulations containing trehalose and dextran.

**KEY WORDS:** β-relaxation; glass transition; insulin; lyophilized formulation; molecular mobility; solid-state stability.

## INTRODUCTION

An increasing number of studies have demonstrated that the chemical stability of drugs in amorphous solids is closely related to matrix mobility. Good correlations between chemical reaction rate and structural relaxation time have been reported for low molecular weight drugs, such as cephalosporins (1), quinapril (2), and aspirin (3), as well as for peptides and proteins, such as a monoclonal antibody (4), IgG1 antibody, and human growth hormone (1,5), and it has been found that the reaction rate constant increases proportionally with structural relaxation time.

On the other hand, other reports show no correlation between chemical stability and matrix mobility. The inactivation of enzymes lyophilized with various excipients is not related to matrix $T_g$ (6–8). The scale of motion required for a given chemical reaction is considered to be one of the most important factors that determine whether chemical stability is correlated with matrix mobility. Chemical reactions that require translational or rotational motion of the entire molecule may exhibit close correlations with matrix mobility, whereas those that require small-scale motion of specific portions of the molecule may not. Recently, β-relaxation, the scale of which is smaller than matrix mobility (α-relaxation), has attracted attention as a molecular motion that determines the stability of amorphous glassy solids more directly than matrix mobility (9). However, little data are available that show direct correlations between chemical stability and β-relaxation.

The purpose of this study is to examine the relationship between chemical stability and β-relaxation in lyophilized insulin formulations, as compared with the relationship between chemical stability and matrix mobility. A previous study with insulin lyophilized from an acidic trehalose

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solution showed that the rates of A21-desamido insulin formation and dimerization via a cyclic intermediate depended on matrix mobility as indicated by $T_g$ under low humidity conditions, but not under high humidity conditions (10). In this study, rotating-frame spin-lattice relaxation time $(T_{1\rho})$, which effectively detects molecular motions on the timescale of $10^{-5}$ s and reflects β-relaxation (11), was measured by $^{13}C$ solid-state NMR for insulin lyophilized with trehalose. Furthermore, the storage stability and β-relaxation of insulin lyophilized with dextran, whose ability to interact with proteins is thought to be less than that of trehalose, were investigated under various temperature and humidity conditions and were compared with those of insulin lyophilized with trehalose, to elucidate the relationship between chemical stability and β-relaxation.

## MATERIALS AND METHODS

### Preparation of Lyophilized Insulin Formulations

Lyophilized insulin formulations containing dextran were prepared as reported (10). Human zinc insulin (Humulin® RU-100) was purchased from Eli Lilly & Co. (Indianapolis, IN, USA) and converted into the zinc-free neutral form by dialysis as reported (12). Dextran 40k (D-4133, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in the zinc-free insulin solution to make a 5 mg/mL solution, and the pH was adjusted to pH 4.0. The resulting solution contained insulin and dextran (1:1.5 w/w). Four hundred microliters of the solution was frozen in a polypropylene sample tube (10 mm in diameter) and then dried at a vacuum level below 5 Pa. Comparison of the amount of insulin before and after freeze drying indicated no significant degradation during freeze drying. Lyophilized samples with various water contents were obtained by storage at 15°C for 24 h in a desiccator with a saturated solution of $LiCl{\cdot}H_2O$ [12% relative humidity (RH)], $K_2CO_3{\cdot}2H_2O$ (43% RH), or $NaBr{\cdot}2H_2O$ (60% RH).

Lyophilized insulin formulations containing trehalose for $T_{1\rho}$, water absorption, and $T_g$ measurements were prepared similarly as the insulin–dextran system, using trehalose (203-02252, Wako Pure Chemical Ind. Ltd, Osaka, Japan) instead of dextran. Similarly, lyophilized insulin alone was prepared without excipients, and lyophilized trehalose alone or dextran alone samples for water absorption and $T_g$ measurements were prepared similarly without insulin.

Similar freeze drying was performed to prepare lyophilized poly-L-glutamic acid (PGA)–dextran or PGA–trehalose samples for $T_{1\rho}$ measurements, using PGA sodium (P-4764, Sigma Chemical Co.) instead of insulin.

### Determination of $T_g$ by Differential Scanning Calorimetry

The $T_g$ of the lyophilized insulin formulations containing dextran was determined by modulated temperature differential scanning calorimetry (2920; TA Instruments, Newcastle, DE, USA), as reported (13). The conditions were as follows: a modulation period of 100 s, a modulation amplitude of ±0.5°C, and an underlying heating rate of 1°C/min. Samples were put in a hermetic pan. Temperature calibration was performed using indium. The samples, preequilibrated at 12, 43, and 60% RH, exhibited a $T_g$ value of 107, 48, and 33°C, respectively. The observed single glass transition suggests that phase separation did not significantly occur during lyophilization.

Similar $T_g$ measurements were carried out for lyophilized insulin alone, trehalose alone, and dextran alone samples preequilibrated at 12% RH.

### Determination of $T_{1\rho}$ of Insulin Carbonyl Carbon by $^{13}C$ Solid-State NMR

The $T_{1\rho}$ of insulin carbonyl carbon in lyophilized insulin, insulin–dextran, and insulin–trehalose samples was determined at 25°C using a UNITY plus spectrometer operating at a proton resonance frequency of 400 MHz (Varian Inc., Palo Alto, CA, USA). Lyophilized samples were preequilibrated at 12% RH. Spin-locking field was equivalent to 19 kHz as a result of amplifier timing out. The rotor size was 7 mm and spinning speed was 4 kHz. Peak height at approximately 180 ppm attributed to insulin carbonyl carbon was followed with delay times of 1, 5, 10, 20, 30, 50, and 80 ms.

Similar measurements of $T_{1\rho}$ were performed for lyophilized PGA alone, PGA–dextran, and PGA–trehalose samples. Signal decay was determined from the area of a slightly split peak around approximately 180 ppm attributed to PGA carbonyl carbon in the backbone and carboxylic carbon in the side chain.

### Water Vapor Absorption

Water vapor absorption isotherms of lyophilized insulin alone, dextran alone, trehalose alone, insulin–trehalose, and insulin–dextran samples were measured gravimetrically at 25°C using an automated vacuum electrobalance (Model MB-300G, VTI Corp., Hialeah, FL, USA). Samples were dried under vacuum until changes in weight were less than 1 μg per 10 min. Water contents of the samples at partial vapor pressures of 0.10 and 0.20 were determined based on equilibrated sample weight (changes in weight of less than 1 μg per 10 min).

### Determination of Insulin Degradation Rate in Lyophilized Formulations Containing Dextran

Lyophilized insulin–dextran samples with various $T_g$ values in tubes with a tight screw cap were stored at a constant temperature (40–90°C), removed at various times, and stored in liquid nitrogen until assayed. Samples were dissolved in 1.5 mL of 0.01 M $(NH_4)_2SO_4$ (pH 2.2, adjusted with concentrated $H_2SO_4$) and subjected to reverse-phase high-performance liquid chromatography (HPLC), as reported (10). The column used was Inertsil WP-300 (C8, 4.6 mm × 250 mm, GL Sciences Inc., Tokyo, Japan) maintained at 35°C. Elutions were performed using a mixture of 0.01 M $(NH_4)_2SO_4$ (pH 2.2) and acetonitrile solution of 0.07%(v/v) trifluoroacetic acid (72.5:27.5) for 1 min. The ratio of the acetonitrile solution increased linearly from 27.5 to 30% in 15 min and from 30 to 35% in 22 min. The detection wavelength was 214 nm.

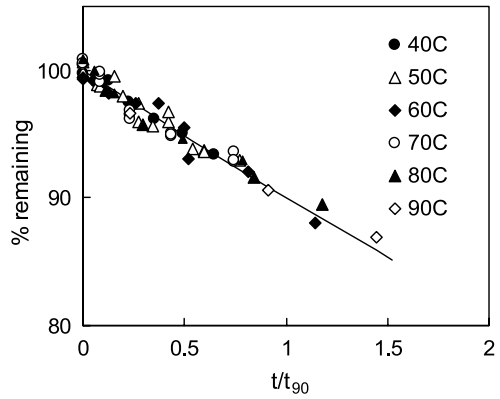


**Fig. 1.** Time courses of insulin degradation in lyophilized formulation containing dextran at 12% relative humidity (RH) at various temperatures. Time is scaled to the $t_{90}$ for each temperature.

## RESULTS

### Insulin Degradation in Lyophilized Formulation Containing Dextran

A21-desamido insulin and insulin dimer were detected by HPLC and size-exclusion chromatography, respectively, as degradation products of lyophilized insulin formulations containing dextran, thus suggesting that the major degradation pathways are A21-desamido insulin formation and dimerization via the cyclic anhydride intermediate, as reported for lyophilized insulin–trehalose and insulin–poly(vinylpyrrolidone) systems (12). The same degradation mechanism has been observed for insulin in acidic solution (14) and in lyophilized solids derived from acidic solutions (15). Figure 1 shows the time courses of insulin degradation at 12% RH in lyophilized formulations containing dextran. Similar time courses were also obtained at 43 and 60% RH. The time courses of insulin degradation determined by HPLC correspond to the formation of the cyclic anhydride intermediate as a rate-determining step. The line shown in Fig. 1 represents the theoretical curve for first-order kinetics. Degradation at initial stages was describable with first-order kinetics under all the temperature and humidity conditions examined. The time required for 10% degradation ($t_{90}$) was calculated from the apparent first-order rate constant.

Figure 2 shows the temperature dependence of the calculated $t_{90}$ for the insulin–dextran system. Solid lines in the figure represent the regression curves obtained by curve fitting according to Eq. (1).

$$k' = k\left(\frac{\alpha' D_r}{k + \alpha' D_r}\right) = k\left(\frac{\alpha T\left(\frac{1}{\tau}\right)^{\xi}}{k + \alpha T\left(\frac{1}{\tau}\right)^{\xi}}\right) = \frac{-\ln(0.9)}{t_{90}} \qquad (1)$$

where $k'$ is the rate constant for a reaction in which the rate-determining step involves molecular diffusion (16). $k$ is the rate constant for conditions under which reactants have high diffusibility, such that $k$ may be described by Eq. (2) using the activation energy ($\Delta H$), frequency factor ($A$), and gas constant ($R$).

$$k = A\ exp\left(\frac{-\Delta H}{RT}\right) \qquad (2)$$

$D_r$ is the diffusion coefficient of the reactant and $\alpha$ ($\alpha'$) is a constant representing the correlation between $D_r$ and reaction rate. $D_r$ was assumed to be related to the structural relaxation time ($\tau$) according to Eq. (3),

$$\frac{D_{r2}}{D_{r1}} \approx \left(\frac{T_2}{T_1}\right)\left(\frac{\tau_1}{\tau_2}\right)^{\xi} \qquad (3)$$

where $\xi$ is a constant that represents the degree of decoupling between $D_r$ and $\tau$ (2). Equation (1) explains that the reaction rate is diffusion-controlled when $k \gg \alpha' D_r$ ($k' = \alpha' D_r$), whereas the reaction rate is controlled by the kinetics of the reaction when $\alpha' D \gg k$ ($k' = k$). The temperature dependence of $k'$ changes around $T_g$ when the reaction rate is diffusion-controlled, but not when the reaction rate is controlled by the kinetics of the reaction. The slope of the regression

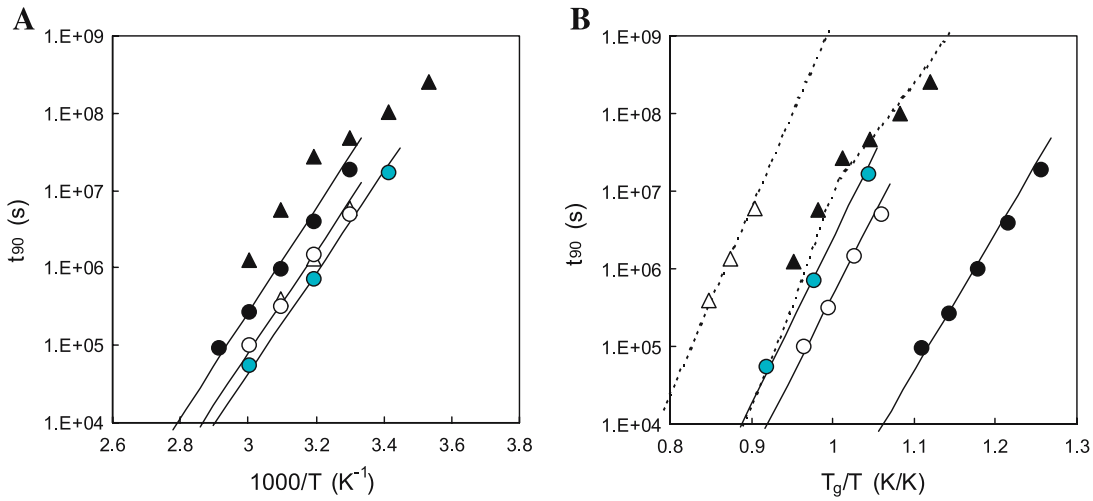


**Fig. 2.** Temperature dependence (A) and $T_g$ dependence (B) of $t_{90}$ for insulin degradation in lyophilized formulation containing dextran or trehalose. ●, dextran 12% RH; ○, dextran 43% RH; ●, dextran 60% RH; ▲, trehalose 12% RH; △, trehalose 43% RH. $t_{90}$ values represent the average of two measurements.

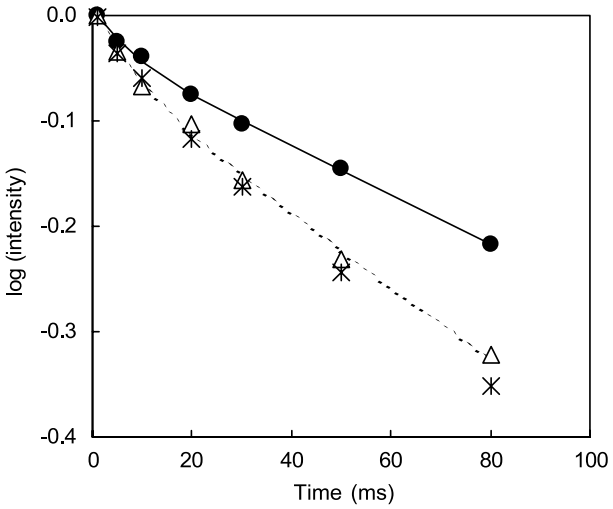


**Fig. 3.** Time courses of rotating-frame spin-lattice relaxation for insulin carbonyl carbon in lyophilized insulin (△), insulin–dextran (∗), and insulin–trehalose (•) systems. 25°C, 12% RH.

curves shown in Fig. 2 did not show significant changes around $T_g$. Therefore, $k'/k$ is considered unity, indicating that the reaction is reaction-controlled. Furthermore, the $t_{90}$ *vs.* $T_g/T$ plots for different humidity conditions did not converge around $T_g$. Curve fitting provided $\Delta H$ estimates of 31.2, 30.5, and 30.0 kcal/mol for 12, 43, and 60% RH, respectively.

Figure 2 also shows the temperature dependence of $t_{90}$ for the insulin–trehalose system reported previously (10) for comparison. As shown in Fig. 2A, $t_{90}$ for the insulin–dextran system at a given temperature was approximately one order of magnitude shorter than that for the insulin–trehalose system at 12% RH, whereas no significant difference in $t_{90}$ was observed between the two systems at 43% RH. Comparison of the $t_{90}$ obtained at 12% RH in the temperature range below the $T_g$ indicates that the insulin–dextran system exhibited a substantially smaller $t_{90}$ value at a given $T_g/T$ than the insulin–trehalose system, as shown in Fig. 2B.

### $T_{1\rho}$ of Insulin Carbonyl Carbon

Figure 3 shows the time courses for the spin-lattice relaxation of insulin carbonyl carbon in lyophilized insulin alone, as well as in lyophilized insulin–dextran and insulin–trehalose systems, observed at 25°C and 12% RH. Spin-lattice relaxation was not significantly affected by the presence of dextran, but it was significantly retarded by the presence of trehalose.

The time course of spin-lattice relaxation was describable with a biexponential equation including two different $T_{1\rho}$ values. Figure 4A and B shows the longer $T_{1\rho}$ estimate and the proportion of the shorter $T_{1\rho}$, respectively, calculated by curve fitting, with the shorter $T_{1\rho}$ being 8 ms. The proportion of the shorter $T_{1\rho}$ was approximately 0.1 regardless of the presence of dextran or trehalose. The longer $T_{1\rho}$ of the dominating proportion, probably attributed to carbonyl carbons in the backbone, was substantially elevated by the presence of trehalose. Under the conditions examined here (slow motional regime), $T_{1\rho}$ is proportional to the correlation time that corresponds to the β-relaxation time (11). Therefore, the increase in the longer $T_{1\rho}$ caused by trehalose suggests that the mobility of carbonyl carbons in the backbone of the insulin molecule is inhibited by trehalose.

To examine the generality of the effects of trehalose and dextran on the $T_{1\rho}$ of carbonyl carbon, $T_{1\rho}$ was measured for PGA carbonyl carbons in lyophilized PGA–trehalose and PGA–dextran systems. The longer $T_{1\rho}$ was substantially increased by the presence of trehalose in a similar manner as the $T_{1\rho}$ of insulin carbonyl carbons. The proportions of the shorter $T_{1\rho}$, probably attributed to carbonyl carbons in the side chains, were estimated to be approximately 0.1 and 0.2 for the insulin–trehalose and PGA–trehalose systems, respectively. In contrast, the proportions of carbonyl carbons present in the side chains are calculated to be 0.09 and 0.5 for insulin and PGA, respectively. This finding suggests that a portion of the carbonyl carbons in the side chain of PGA exhibits low mobility comparable to the carbonyl carbons in the backbone of the molecule.

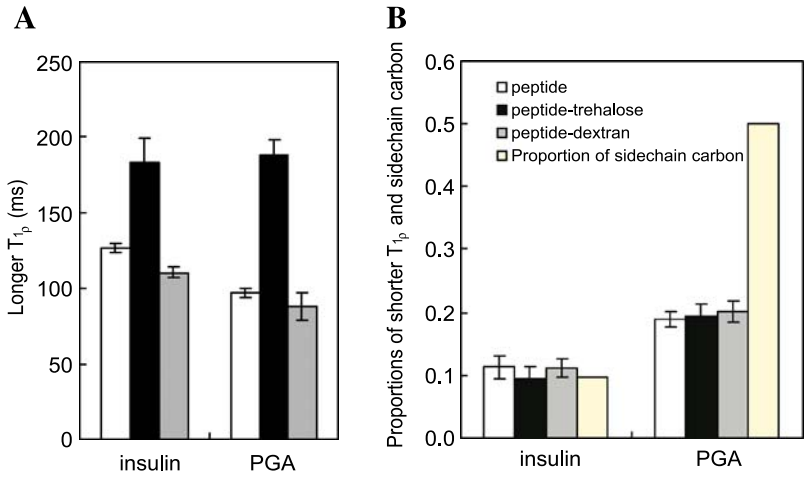


**Fig. 4.** Effect of trehalose and dextran on the $(T_{1\rho})$ of insulin and poly-L-glutamic acid carbonyl carbons. Signal decay was analyzed by a biexponential equation. The longer $(T_{1\rho})$ and the proportion of shorter $(T_{1\rho})$ are shown in (A) and (B), respectively. (B) also shows the proportion of carbons in the side chain.

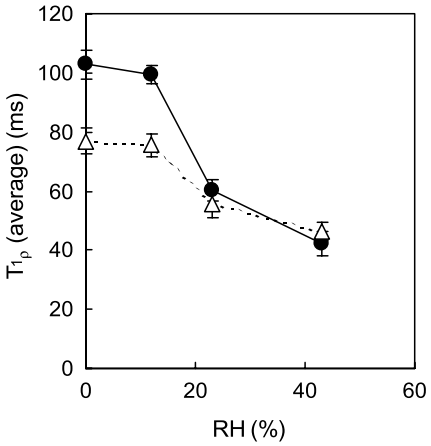


**Fig. 5.** Effect of humidity on the $(T_{1\rho})$ of insulin carbonyl carbon in the backbone carbon in lyophilized insulin (△) and insulin–trehalose (•) systems. 25°C. Bars represent standard deviation ($n$ = 3).

Figure 5 shows the effects of humidity on the longer $T_{1\rho}$ of insulin carbonyl carbons. The prolongation of $T_{1\rho}$ brought about by the addition of trehalose was eliminated at above 23% RH, suggesting that trehalose inhibits the mobility of insulin carbonyl carbons in the backbone only under low humidity conditions.

In an effort to elucidate the relationship between the changes in $T_{1\rho}$ and in matrix $T_g$ associated with the addition of excipients, the amount of absorbed water at 10 and 20% RH as well as matrix $T_g$ at 12% RH were measured for the lyophilized insulin alone, trehalose alone, dextran alone, insulin–trehalose, and insulin–dextran systems, and the results are shown in Table I. The insulin–dextran system exhibited a similar amount of absorbed water as dextran alone and exhibited an intermediate $T_g$ value between those of insulin alone and dextran alone. Meanwhile, the insulin–trehalose system exhibited a larger amount of absorbed water and a lower $T_g$ than insulin alone and trehalose alone. These findings indicate that the addition of trehalose increases water absorption, resulting in decreased matrix $T_g$.

## DISCUSSION

Our previous study demonstrated that the degradation rate of insulin in a lyophilized insulin–trehalose system is significantly affected by matrix $T_g$ under low humidity conditions, but not under higher humidity conditions, based on curve fitting for the temperature dependence of $t_{90}$ according to Eq. (1) (dotted lines in Fig. 2B). The present study demonstrated that the temperature dependence of $t_{90}$ for a lyophilized insulin–dextran system did not exhibit significant changes in the slope of the regression curve around the $T_g$, even under low humidity conditions. The $t_{90}$ of insulin degradation in the insulin–trehalose system was approximately one order of magnitude greater than that in the insulin–dextran system at 12% RH, but the difference was not significant at 43% RH (Fig. 2A). Although dextran was expected to stabilize insulin because of the high $T_g$ value (matrix $T_g$ of the insulin–dextran and insulin–trehalose systems at 12% RH was 107 and 44°C, respectively), the insulin–dextran system exhibited a substantially shorter $t_{90}$ than the insulin–trehalose system at a given $T_g/T$ (matrix mobility) (Fig. 2B). This finding suggests that it is not matrix mobility that determines the stability of insulin. This notion is supported by the finding that the temperature dependence of $t_{90}$ for the insulin–dextran systems with a different $T_g$ caused by a different water content did not converge around $T_g$ as expected when matrix mobility is a determinant of degradation rate.

Other than the matrix mobility of amorphous solids, another motion suggested to determine the stability of the solids is β-relaxation, a smaller-scale motion than matrix mobility (9). It is widely known that $T_{1\rho}$ can effectively detect molecular motions on the timescale of $10^{-5}$ s and reflect the β-relaxation of molecules in amorphous solids (11,17,18). The present study demonstrated that the $T_{1\rho}$ of insulin carbonyl carbons under low humidity conditions was substantially increased by the presence of trehalose, but not by the presence of dextran (Fig. 4). This increase in $T_{1\rho}$ indicates that trehalose retards the β-relaxation of insulin. No effect of dextran on the $T_{1\rho}$ suggests that miscibility between insulin and dextran is lower than that between insulin and trehalose. Meanwhile, the addition of trehalose brought about a decrease in $T_g$ or an increase in matrix mobility (Table I). The behaviors of $T_{1\rho}$ and $T_g$ described above suggest that the β-relaxation of insulin molecules rather than the matrix mobility is inhibited by trehalose. The addition of trehalose improved the stability of insulin and inhibited the β-relaxation of insulin under low humidity conditions, whereas the addition of dextran did not exhibit significant effects on the stability nor on the β-relaxation (Figs. 2 and 4). Therefore, the β-relaxation of insulin rather than the matrix mobility seems to be closely related to the stability of insulin. Close correlations between β-relaxation and stability are also suggested by the findings that the β-relaxation-inhibiting effects of trehalose were eliminated at above 23% RH (Fig. 5) and that the stabilizing effect of trehalose was eliminated at 43% RH (Fig. 2A). The β-relaxation-inhibiting effect of trehalose may be attributed to the ability of trehalose to interact with insulin through hydrogen bonding. It is well known that sugars such as trehalose improve the storage stability of lyophilized protein formulations regardless of their relatively low $T_g$ values (1). This stabilizing effect is generally attributed to interaction between sugar and protein.

The degradation rate of insulin under low humidity conditions was significantly affected by matrix $T_g$ in lyophilized insulin–trehalose systems, as reported previously (10), but was not significantly affected by matrix $T_g$ in the insulin–dextran systems. These findings suggest that the β-

**Table I.** Effect of Trehalose and Dextran on Water Content and $T_g$

| | Water content (%/solid) | | |
|---|---|---|---|
| | 10% RH | 20% RH | $T_g$ at 12% RH |
| Insulin | 2.8 ± 0.1 | 3.6 ± 0.1 | 106 ± 2 |
| Trehalose | 3.3 ± 0.0 | 4.8 ± 0.0 | 66 ± 1 |
| Dextran | 5.5 ± 0.5 | 7.6 ± 0.7 | 131 ± 4 |
| Insulin–trehalose | 4.3 ± 0.1 | 6.5 ± 0.0 | 48 ± 3 |
| Insulin–dextran | 5.6 ± 0.2 | 7.8 ± 0.2 | 107 ± 3 |

SD ($n$ = 3).
RH = relative humidity.

relaxation of insulin is inhibited by trehalose and, thus, becomes a rate determinant, while it is not inhibited by dextran, and the chemical activational barrier (activation energy) becomes the major rate determinant.

$D_r$ in Eq. (1) is related to the structural relaxation time ($\tau$), which reflects matrix mobility, according to Eq. (3). Therefore, it was previously concluded that the degradation rate of insulin at low humidities in the insulin–trehalose system is affected by matrix mobility. However, it may also be possible that β-relaxation time is a determinant of degradation rate, such that $\tau$ in Eq. (1) represents β-relaxation time. The temperature dependence of the degradation rate at low humidities, which exhibits a change in slope around $T_g$, can be explained by assuming that β-relaxation is coupled with matrix mobility. It is generally understood that smaller-scale motions can be coupled with matrix mobility (5,18,19). The thought that β-relaxation rather than matrix mobility determines the degradation rate is reasonable because insulin degradation via a cyclic intermediate is not believed to require large-scale motions, such as matrix mobility (5).

## CONCLUSION

The storage stability and β-relaxation of insulin lyophilized with trehalose were compared with those of insulin lyophilized with dextran. The addition of trehalose improved the stability of insulin and inhibited the β-relaxation of insulin under low humidity conditions, while the addition of dextran did not exhibit significant effects on stability nor on β-relaxation. The β-relaxation-inhibiting and the stabilizing effects of trehalose were eliminated at higher humidities. These results suggest that β-relaxation of insulin is inhibited by trehalose at low humidity, presumably as a result of insulin–trehalose interaction, and thus, β-relaxation is a determinant of the degradation rate of insulin. In contrast, the β-relaxation of insulin is not inhibited by dextran, and thus, the chemical activational barrier (activation energy) is the major rate determinant. The molecular motion related to the stability of insulin in the present glassy systems seems to be the β-relaxation of insulin rather than the matrix mobility.

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