Pharmaceutical Research, Vol. 14, No. 6, 1997

Dependence of the Molecular Mobility and Protein Stability of Freeze-Dried γ-Globulin Formulations on the Molecular Weight of Dextran

Sumie Yoshioka,[1,2] Yukio Aso,[1] and Shigeo Kojima[1]

Received January 13, 1997; accepted March 6, 1997

Purpose. The effect of the molecular weight of dextran on the molecular mobility and protein stability of freeze-dried serum γ-globulin (BGG) formulations was studied. The stabilizing effect of higher molecular weight dextran is discussed in relation to the molecular mobility of the formulations.

Methods. The molecular mobility of freeze-dried BGG formulations containing dextrans of various molecular weights was determined based on the free induction decay of dextran and water protons measured by proton NMR. The protein stability of the formulations was determined at temperatures ranging from 20 to 70°C by size exclusion chromatography.

Results. Changes in the molecular mobility of freeze-dried formulations that occurred at temperatures below the glass transition temperature could be detected as the molecular mobility-changing temperature (T_{mc}), at which dextran protons started to exhibit a Lorentzian relaxation decay due to higher mobility in addition to a Gaussian relaxation decay. T_{mc} increased as the molecular weight of dextran increased. The proportion of dextran protons which exhibited the higher mobility relaxation process (P_{hm}) at temperatures above T_{mc} decreased as the molecular weight of dextran increased. Protein stability was closely related to molecular mobility. The temperature dependence of the denaturation rate changed at around T_{mc}, and denaturation in the microscopically liquidized state decreased as P_{hm} decreased with increasing molecular weight of dextran.

Conclusions. The effect of the molecular weight of dextran on the protein stability of freeze-dried BGG formulations could be explained in terms of the parameters obtained by ^{1}H-NMR such as T_{mc} and P_{hm}. These parameters appear to be useful in preformulation and stability prediction of freeze-dried formulations.

KEY WORDS: molecular mobility; spin-spin relaxation time; protein stability; dextran; freeze-drying.

INTRODUCTION

The stability of lyophilized proteins during storage strongly depends on water content and temperature (1–4), which appear to influence protein stability by changing the molecular mobility of lyophilized cakes. Studies have shown that the water content- and temperature-dependence of the stability of lyophilized bovine serum albumin (BSA) and γ-globulin (BGG) is closely related to changes in molecular mobility as measured by the spin-spin relaxation time (T_2) of protein protons (5,6). An increase in molecular mobility observed with increasing water content was associated with a decrease in protein stability. At higher water contents, stability increased with decreasing molecular mobility, as water content increased (5). A sudden increase in the molecular mobility of lyophilized BSA and BGG at the softening temperature, determined from the abrupt change in the T_2 of the protein protons ($T_{s(T2)}$), resulted in a sudden decrease in protein stability (6). The results of studies using lyophilized BSA and BGG that contained no excipient indicate that the molecular mobility determined by T_2 is a useful indicator of protein stability. It is of great interest to investigate whether a similar relationship exists in freeze-dried protein formulations with excipients.

The addition of excipients with a high glass transition temperature (T_g) is believed to increase the stability of freeze-dried formulations during storage (2,7). However, few studies have isolated the stabilizing effects due to high T_g separately from those due to other chemical and physical properties of excipients. Another area of great interest is the stabilizing effect that results from the high T_g of excipients.

The present study determined the molecular mobility of freeze-dried BGG formulations that contained dextrans of various molecular weights by ^{1}H-NMR relaxation measurement. The effect of the molecular weight of dextran on the molecular mobility and protein stability of formulations was examined. Furthermore, the stabilizing effect due to high T_g is discussed in relation to the molecular mobility of the formulations. Finally, the usefulness of ^{1}H-NMR for evaluating the molecular mobility of freeze-dried formulations containing excipients is also discussed. In a previous paper (6), we determined the T_2 of freeze-dried proteins as a parameter to represent the molecular flexibility, and defined $T_{s(T2)}$ as the microscopic softening temperature determined from changes in the T_2. However, the terms "molecular flexibility" and "softening temperature" have been considered to be ambiguous. Therefore, because T_1 and T_2 are parameters to represent molecular mobility (8,9), $T_{s(T2)}$ was replaced by a new parameter, the molecular mobility-changing temperature (T_{mc}).

MATERIALS AND METHODS

Preparation of Lyophilized BGG Formulations

Ten milligrams of BGG (G5009, Sigma Chemical Co., St. Louis, MO) and dextrans (molecular weights of 10 k, 40 k, 70 k, and 510 k, Sigma Chemical Co.) were dissolved in distilled water (6.64 g). Dextran concentration was 7%w/w. Three hundred microliters of the solution was frozen in a polypropylene sample tube (10 mm diameter) by immersion in liquid nitrogen for 10 min, and then dried at a vacuum level below 5 Pa for 23.5 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo), as previously described (5,6).

The lyophilized samples were stored at 15°C in a desiccator with either 23.4 or 60.2% relative humidity (RH) (adjusted with saturated solutions of potasium acetate and NaBr, respectively) for 24 h in order to establish the desired water content. Water content was determined by the Karl Fisher method (684 KF Coulometer, Switzerland). Water content after storage at 23.4%RH was 0.086, 0.089, 0.090, and 0.088g/g solid for the samples containing dextrans of molecular weights of 10 k, 40 k, 70 k, and 510 k, respectively. Water content after storage at 60.2%RH was 0.16, 0.17, 0.18, and 0.18g/g solid for the samples

---

[1] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

[2] To whom correspondence should be addressed. (e-mail: yoshioka@nihs.go.jp)

0724-8741/97/0600-0736$12.50/0 © 1997 Plenum Publishing Corporation

with 10 k, 40 k, 70 k, and 510 k, respectively. No significant differences in water content were observed among formulations containing dextrans of different molecular weights. Water content after freeze-drying and before water content adjustment was less than 0.01 g/g solid for all samples.

^{1}H NMR Measurement

The free induction decay (FID) of protons in the freeze-dried BGG formulations stored at 23.4 and 60.2%RH were obtained at temperatures ranging from 10 to 90°C, using a pulse NMR spectrometer (25 MHz, JNM-MU25, JEOL, Tokyo), as previously described (5,6). The 90° pulses were a width of 2 μs and a recycling time of 3 s. 'Solid echo' with an echo delay of 10 μs was used in the detection stage.

The T_2 of water and dextran protons in freeze-dried formulations was calculated from the FID. For the formulations stored at 60.2%RH, the T_2 of water protons was calculated using FID signals between 100 and 1000 μs by non-linear least-squares regression analysis according to the Lorentzian equation ($\exp(-t/T_2)$). For the formulations of lower water content stored at 23.4%RH, the T_2 of water protons at 30 and 50°C was calculated from FID signals between 100 and 300 μs, and those between 100 and 700 μs, respectively. FID signals between 100 and 1000 μs were used for the T_2 at 70 and 90°C.

FID signals due to dextran protons were described by the Abragam equation (Eq. 1), which is a Gaussian-type equation, as shown in Fig. 1.

$$F(t) = \exp\left(-t^2/2T_2^2\right) \sin(at)/at \qquad (1)$$

where a is a constant (10). The T_2 of dextran protons was calculated from FID signals between 2.6 and 100 μs by inserting the calculated T_2 of water protons into an equation representing the sum of the Abragam and Lorentzian equations. Constant a was 0.12 for all the samples.

Differential Scanning Calorimetry (DSC)

Thermograms of freeze-dried BGG formulations were obtained at a scan rate of 10°C/min by calorimetry (DSC 2920, TA Instruments, New Castle, DE). Five milligrams of each sample was hermetically sealed in an aluminum pan.

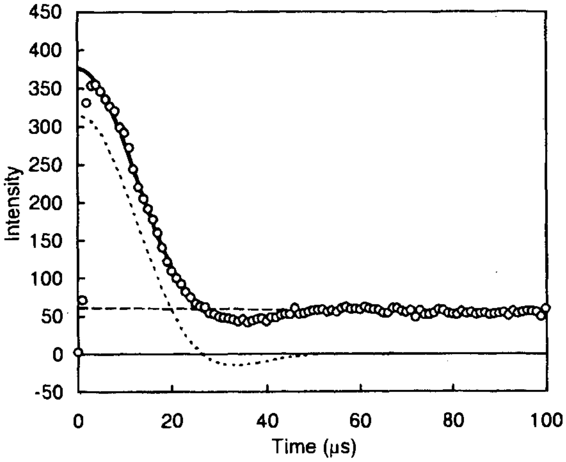

Fig. 1. On-resonance FID signals obtained from a freeze-dried BGG formulation containing MW 10 k dextran with a water content of 0.09g/g solid at 90°C. ---- : Lorentzian decay of water protons --- : Gaussian-type (Abragam) decay of dextran proton.

Determination of BGG Denaturation by High Performance Size Exclusion Chromatography

The stability of the freeze-dried BGG formulations that contained dextran of various molecular weights, which had been stored at either 23.4 or 60.2%RH in order to adjust the water content, was determined at temperatures ranging from 20 to 70°C. The remaining intact proteins after the 20 h-storage period were measured by size exclusion chromatography as previously described (5,6). The column (Tosoh G3000SW, 30 cm × 7.5 mm, Tokyo) was maintained at 30°C and 200 mM phosphate buffer (pH 6.2) was used as the mobile phase.

RESULTS

Effect of Molecular Weight of Dextran on Protein Denaturation

Figure 2 shows the typical size exclusion chromatograms of freeze-dried BGG formulations that contained dextrans of various molecular weights before and after storage at 60°C. No significant change in the total peak area was observed after storage, indicating that insoluble aggregates were not formed during storage. This may result from inhibition of protein aggregation by dextran molecules surrounding the protein molecules. A decrease in the peak height due to intact protein was observed at approximately 420 seconds, and an increase in peak height due to denatured and aggregated proteins was observed at shorter retention times during storage. Freeze-dried formulation that contained 10 k molecular weight (MW) dextran exhibited a peak due to denatured protein that overlapped with the peak due to intact protein. The largest increase in intensity during storage was observed at a retention time that decreased as the molecular weight of dextran increased. It is speculated that denatured protein was eluted in a form bound to dextran at a retention time determined by the molecular weight of dextran (11). The retention time of the peak due to intact protein before storage was not affected by the molecular weight of dextran, indicating that intact protein was not bound to dextran.

Figure 3 shows the peak height of intact proteins determined for freeze-dried BGG formulations that contained dextrans of various molecular weights after storage at 60°C. The peak height determined before and after lyophilization, as well as after adjusting the water content is also shown in Fig. 3. The peak ratio is represented as a ratio to the peak height of BGG standard solution without dextran. The standard solution contained denatured and aggregated protein, such that the peak area of intact protein in the standard solution was calculated to be 82%. Although denaturation was observed during freeze-drying and water content adjustment, the effect of the molecular weight of dextran was not significant. For the MW 510 k dextran formulation, the peak due to intact protein in the solution prior to freeze-drying was significantly lower (Fig. 3) and the peak due to denatured (aggregated) protein at 300s was larger than that for the other formulations (Fig. 2). This may be due to denaturation upon the dissolution of protein in the viscous solution of high molecular weight dextran. Storage at 60°C caused more marked denaturation, the degree of which

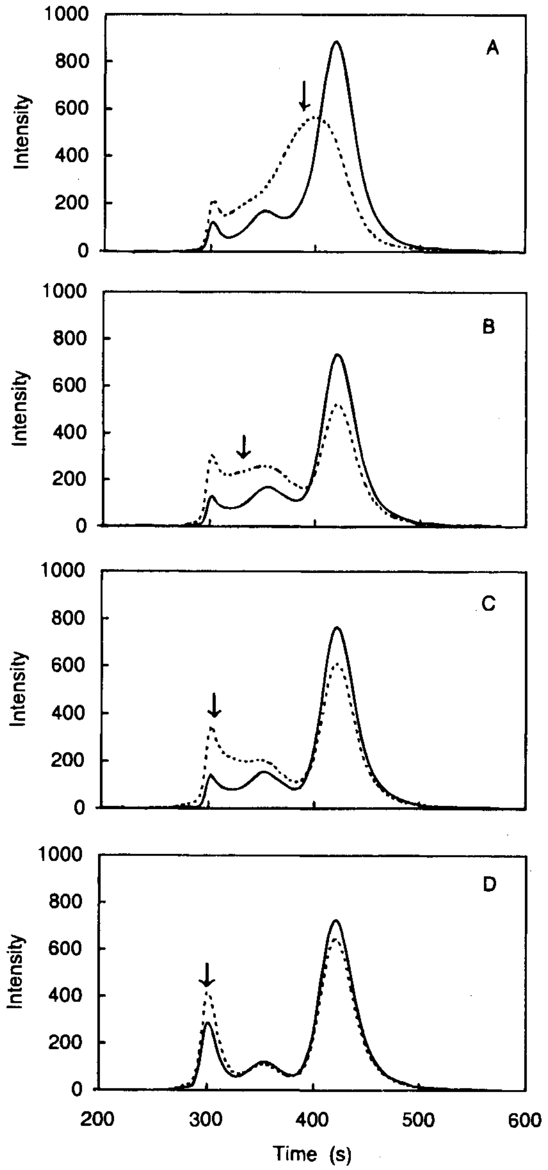

Fig. 2. Size exclusion chromatograms of freeze-dried BGG formulations containing dextran of various molecular weights before (—) and after (---) storage at 60°C for 20 h. Molecular weight of dextran: 10 k (A); 40 k (B); 70 k (C); 510 k (D). Water content: 0.09g/g solid. Arrows indicate the largest increase in intensity during storage.

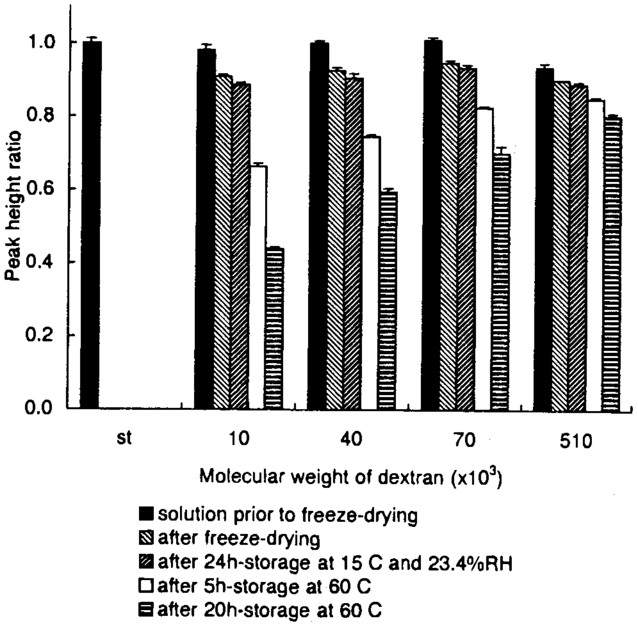

Fig. 3. Peak height ratio of intact protein in freeze-dried BGG formulations containing dextran of various molecular weights. St represents BGG standard solution without dextran. Water content: 0.09 g/g solid.

depended on the molecular weight of dextran. Dextran of a smaller molecular weight demonstrated a higher degree of protein denaturation. The freeze-dried protein formulation that contained MW 10 k dextran was found to be the most unstable, although the peak height due to intact protein in this formulation was overestimated as a result of overlapping with the peak due to denatured protein, as shown in Fig. 2.

The effect of the molecular weight of dextran on BGG stability shown in Figs. 2 and 3 was observed in the formulation with a water content of 0.09g/g solid. A similar effect was observed in the formulation with a water content of 0.17g/g solid. Figure 4 shows the degree of denaturation at 0.09 and 0.17 g/g solid as a function of temperature. At both water contents, the formulation that contained MW 10 k dextran exhibited a higher degree of denaturation than that containing MW 510 k dextran.

Molecular Mobility of Freeze-Dried Formulations Determined by Proton NMR

The FID of protons present in the freeze-dried BGG formulation was obtained in order to determine the molecular mobility. Figure 5 shows the FID obtained for the formulation of MW 10 k dextran with a water content of 0.17g/g solid. A slower decay was described by the Lorentzian equation, and this is attributed to water protons. In contrast, faster decay can be ascribed primarily to the dextran protons, because the content of dextran in the formulations was 50 times larger than that of

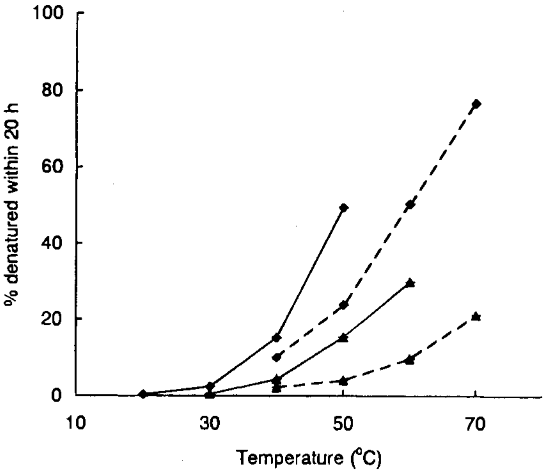

Fig. 4. Denaturation of freeze-dried BGG formulations of MW 10 k (♦) and 510 k (▲) dextran as a function of temperature. Water content: 0.09 (----); 0.17 g/g solid (—).

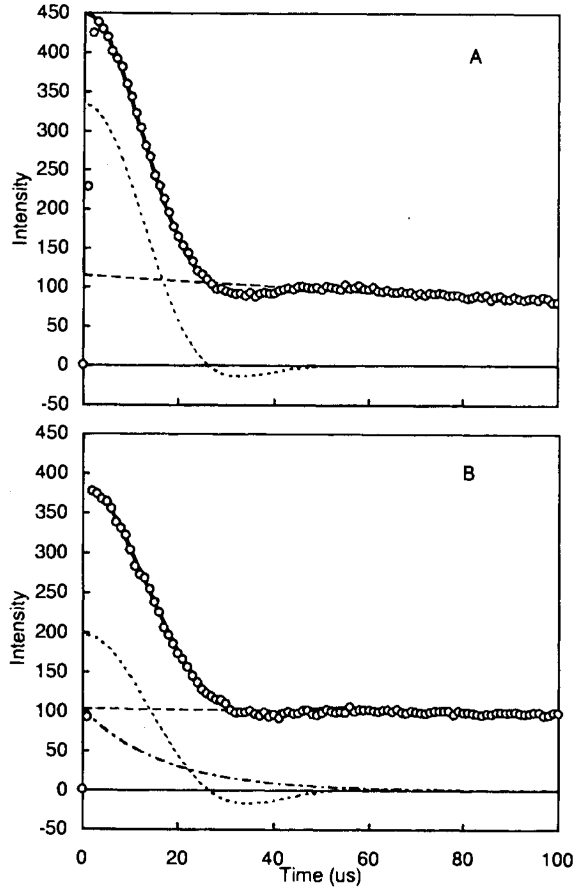

Fig. 5. On-resonance FID signals obtained from a freeze-dried BGG formulation containing MW 10 k dextran with a water content of 0.17 g/g solid at 10°C (A) and 60°C (B). ——: Lorentzian decay of H_2O protons ---: Gaussian-type (Abragam) decay of dextran proton — - —: Lorentzian decay of dextran proton.

protein. The decay due to dextran protons at 10°C was described by the Gaussian-type equation that can fit the relaxation process of solid protons, whereas that at 60°C could not be described by single Gaussian-type decay, and therefore required further solving by the Lorentzian equation that describes the relaxation process of liquid protons. This indicates that the dextran protons of the freeze-dried formulations exhibited a Lorentzian relaxation process due to higher mobility in addition to a Gaussian-type relaxation process due to lower mobility at 60°C.

Figure 6-A shows the T_2 of dextran protons calculated by the Gaussian-type equation as a function of temperature. When the Lorentzian decay of dextran protons was detected, the proportion of dextran protons exhibiting the higher mobility relaxation process (P_{hm}) was calculated, as shown in Fig. 6-A. At a water content of 0.17 g/g solid, a higher mobility relaxation process with a T_2 of approximately 17 μs was detected at temperatures of 40°C and higher. P_{hm} was higher for the formulation of MW 10 k dextran than for that of MW 510 k dextran. At a water content of 0.09g/g solid, the formulation exhibited only Gaussian-type decay, even at 90°C, as shown in Fig. 1. In this case, higher mobility relaxation processes of dextran protons were not detected. No significant differences in the calculated T_2 were observed between the formulations of MW 10 k and 510 k dextran (Fig.6-A).

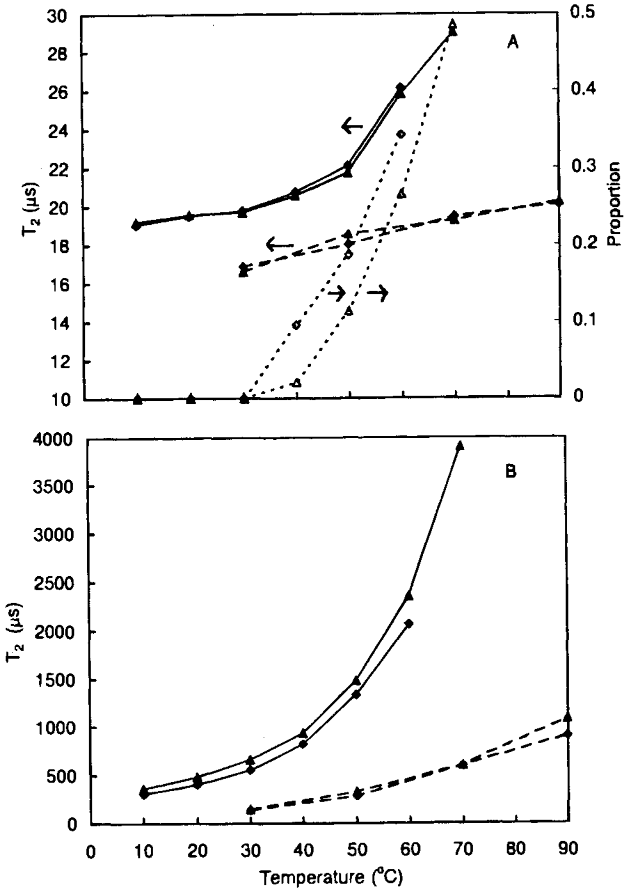

Fig. 6. Spin-spin relaxation time of dextran protons (A) and water protons (B) in freeze-dried BGG formulations, and proportion of dextran protons exhibiting the higher mobility relaxation process.
—◆—: T_2 of formulation of MW 10 k dextran with a water content of 0.17g/g solid
—▲—: T_2 of formulation of MW 510 k dextran with a water content of 0.17g/g solid
--◆--: T_2 of formulation of MW 10 k dextran with a water content of 0.09g/g solid
--▲--: T_2 of formulation of MW 510 k dextran with a water content of 0.09g/g solid
----◇----: proportion of MW 10 k dextran protons exhibiting the higher mobility relaxation process with a water content of 0.17g/g solid
----△----: proportion of MW 510 k dextran protons exhibiting the higher mobility relaxation process with a water content of 0.17g/g solid.

Figure 6-B shows the T_2 of water protons calculated by the Lorentzian equation as a function of temperature. At a water content of 0.09 and 0.17g/g solid, the T_2 of water protons was not affected significantly by the molecular weight of dextran.

Transition of Freeze-Dried BGG Formulations from Non-Liquidized Solid to Microscopically Liquidized Solid

The sudden change in molecular mobility observed at 40°C (Fig. 6-A) suggests that a formulation with a water content of 0.17g/g solid exhibits a transition from non-liquidized solid to microscopically liquidized solid at around 40°C. The denaturation rate of the freeze-dried BGG formulations of MW 40 k and 510 k dextran with a water content of 0.17g/g solid was determined at around that temperature. The data appear in Fig. 7-A. The parameters that demonstrate molecular mobility as determined by NMR are shown in Fig. 7-B. As shown in Fig. 7-A, the denaturation rate of both formulations of MW 40 k and 510 k dextran exhibited a discontinuity at around 40°C. This may be due to the transition from a non-liquidized state at lower temperatures to a microscopically liquidized state at higher temperatures. The formulation of MW 40 k dextran showed a discontinuity at a lower temperature than did MW 510 k dextran.

As shown in Fig. 7-B, the T_2 of dextran protons was similar for both formulations of MW 40 k and 510 k dextran. The T_2 increased in a Arrhenius fashion with an increase in temperature at temperatures below 40°C. At 40°C, the higher mobility relaxation process of dextran protons was apparent and the increase in T_2 became divergent from the linear Arrhenius relationship. This T_2 change may be fitted better by equations representing parameters around phase transitions such as the Williams, Landel and Ferry (WLF) equation. P_{hm} was higher for the formulation of MW 40 k dextran than for that of MW 510 k dextran.

The "softening temperature ($T_{s(T2)}$)", defined in a previous paper as the temperature at which a higher mobility relaxation process of protein protons started to appear, can be defined better as the molecular mobility-changing temperature (T_{mc}), since the temperature is determined from the change in the T_2 which represents molecular mobility. The T_{mc} of the formulations that contained dextran studied in the present study was determined to be approximately 40°C (Fig. 7-B). This value is consistent with the transition temperature as determined from the discontinuity of the Arrhenius plots of denaturation rate (Fig. 7-A).

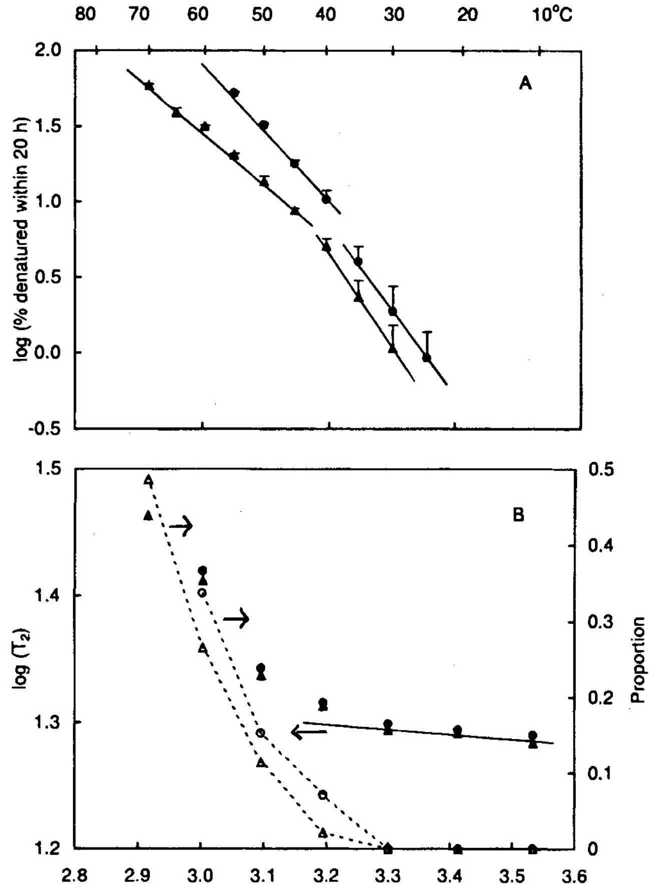

Fig. 7. Temperature dependence of denaturation rate (A), and of spin-spin relaxation time of dextran protons (●▲) and proportion of dextran protons exhibiting the higher mobility relaxation process (○△) (B) of freeze-dried BGG formulation with a water content of 0.17g/g solid. Molecular weight of dextran: 40 k (●○); 510 k (▲△).

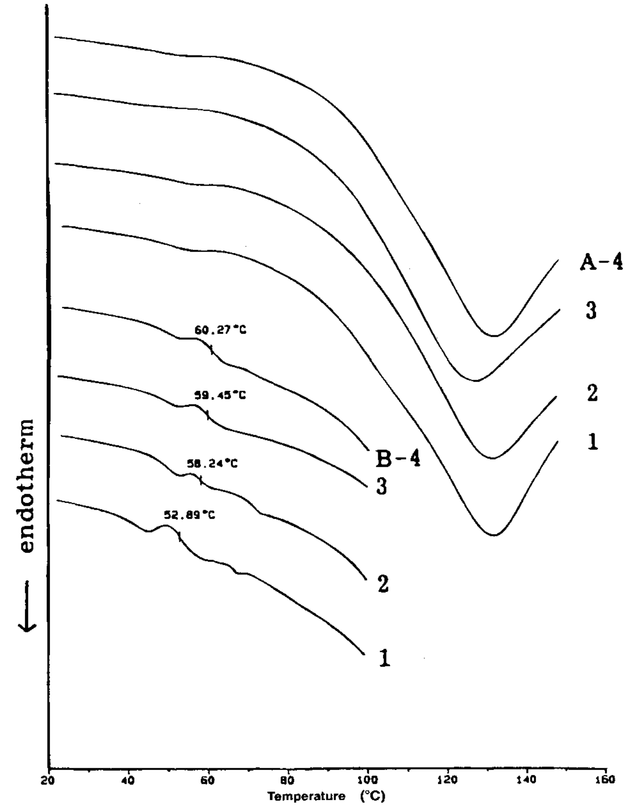

Fig. 8. Differential scanning calorimetry thermograms for freeze-dried BGG formulations with water contents of 0.09 (A) and 0.17g/g solid (B). Molecular weight of dextran: 10 k (1), 40 k (2), 70 k (3), and 510 k (4).

For comparison with T_{mc}, the T_g of the freeze-dried BGG formulations was determined by DSC. As shown in Fig. 8, the T_g of the formulation with a water content of 0.17g/g solid was higher than the T_{mc} determined by NMR. This indicates that T_{mc} determined by NMR represents the temperature at which the molecular mobility begins to increase in a temperature range lower than the T_g determined by DSC. The formulation with a water content of 0.09g/g of solid showed almost no change in heat capacity up to 90°C. This finding is consistent with the finding that the T_{mc} was not detected at a temperature below 90°C.

DISCUSSION

The rate of protein denaturation during storage of freeze-dried formulations is determined by the free energy of activation, ΔG*. The difference in protein stability observed among the freeze-dried BGG formulations that contained dextran of different molecular weights (Figs. 3, 4, and 7-A) may be ascribed to the difference in ΔG* resulting from the different molecular mobility of the formulations, rather than due to differences in BGG-dextran interaction. Even if some interaction occurs between activated BGG and dextran as suggested by the binding of denatured BGG to dextran (Fig. 2), alternation of the interaction leading to different ΔG^* is not believed to be induced by different molecular weights of dextran, because the interaction is determined by the properties of the dextran monomer unit. This notion is supported by the lack of effect of molecular weight of dextran on water-dextran interaction as evidenced by the lack of significant differences in the mobility of water molecules in the formulation (Fig. 6-B). The difference in molecular mobility appears to be more responsible for the difference in stability associated with differing molecular weight of dextran. ΔG^* may depend on the molecular mobility of the formulations, such that ΔG^* is smaller in formulations of high molecular mobility (microscopically liquidized state) than in formulations of low molecular mobility (non-liquidized state). Thus, formulations of high molecular mobility exhibit less stability during storage.

Freeze-dried BGG formulations that contained dextran of different molecular weights exhibited a molecular mobility-changing temperature at around 40°C, representing the transition from a non-liquidized to a microscopically liquidized state (Figs. 6-A and 7-B). Formulations that contain dextran of a higher molecular weight have a higher T_{mc} and transform into a microscopically liquidized state at a higher temperature, resulting in greater stability.

The proportion of dextran protons exhibiting a higher molecular relaxation process at temperatures above T_{mc} was also affected by the molecular weight of dextran, such that a lower proportion of formulations with dextran of a higher molecular weight was evident at the same temperature. This indicates that the molecular mobility of formulations in the microscopically liquidized state decreases as the molecular weight of dextran increases. The observed difference in molecular mobility may be explained by the smaller ratio of higher mobility polymer chain terminal groups, and/or by the lower mobility of higher molecular weight dextran molecule.

Protein stability was closely related to the molecular mobility. The temperature dependence of the denaturation rate changed at around T_{mc} (Fig. 7). Denaturation in the microscopically liquidized state was less in formulations with a higher molecular weight dextran which showed a lower P_{hm} (Figs. 4 and 8). Thus, T_{mc} and P_{hm} determined from the FID are considered to be important indicators of protein stability in freeze-dried formulations. T_{mc}, which represents the temperature at which the molecular mobility of formulations starts to increase significantly, appears to be a more sensitive parameter that is more closely related to protein stability than T_g determined by DSC. Determination of T_g is generally difficult for freeze-dried protein formulations that contain various excipients due to the heterogeneous physical state. As a result, the T_g of individual excipients has usually been reported (7).

The higher mobility relaxation process of dextran protons was not detected in formulations with a water content of 0.17 and 0.09g/g solid at temperatures below 30°C and 90°C, respectively. This indicates the formulations exist in the non-liquidized state at these temperatures. T_2 increased with increasing temperature in a Arrhenius fashion in the non-liquidized state. No difference in T_2 was observed among formulations of different molecular weight dextrans, indicating that there was no difference in the molecular mobility determined by NMR (Figs. 6-A and 7-B). However, the protein denaturation rate was affected by dextran molecular weight even in the non-liquidized state, such that less denaturation was evident in formulations of higher molecular weight dextran (Figs. 3, 4, and 7-A). The difference in protein stability in the non-liquidized state associated with the different molecular weight of dextran suggests that there are differences in the molecular mobility among formulations of different molecular weight dextrans. However, this difference was not evident in the T_2 of dextran protons. Therefore, it is speculated that the difference in molecular mobility in a extremely low range associated with the different molecular weights of dextran cannot cause a significant difference in T_2 since molecular mobility in the non-liquidized state is much lower than that in the microscopically liquidized state. This appears to be a limitation of NMR for determining the molecular mobility of formulations with very low molecular mobility.

CONCLUSIONS

The effect of the molecular weight of dextran on the protein stability of freeze-dried BGG formulation could be explained in terms of T_{mc} and P_{hm}. The stabilizing effect of dextran of a higher molecular weight occurs as a result of a higher T_{mc} and a smaller P_{hm} in the microscopically liquidized state. The change in molecular mobility of freeze-dried formulations that occured at temperatures below the T_g could be detected at temperatures around T_{mc}, and protein stability was more closely related to T_{mc} than T_g. Thus, the parameters obtained by proton NMR appear to be useful in preformulation and stability prediction of freeze-dried formulations that contain excipients.

REFERENCES

1. M. Hageman. *Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation,* Plenum Press, New York, 1992.
2. L. N. Bell, M. J. Hageman, and L. M. Muraoka. *J. Pharm. Sci.* **84**:707–712 (1995).
3. F. Franks. *Jpn. J. Freezing Drying.* **38**:5–16 (1992).
4. M. Pikal. *J. Biopharm.* **3**:26–30 (1990).
5. S. Yoshioka, Y. Aso, and S. Kojima. *Pharm. Res.* **13**:926–930 (1996).
6. S. Yoshioka, Y. Aso, and S. Kojima. *J. Pharm. Sci.* **86**:470–474 (1997).
7. S. J. Prestrelski, K. A. Pikal, and T. Arakawa. *Pharm. Res.* **12**:1250–1259 (1995).
8. C. A. Oksanen and G. Zografi. *Pharm. Res.* **10**:791–799 (1993)
9. M. T. Kalichevsky, E. M. Jaroszkiewicz, A. Ablett, J. M. V. Blanshard, P. J. Lillford. *Carbohydrate Polymers.* **18**:77–88 (1992).
10. N. Parizel, G. Meyer, and G. Weill. *Polymer,* **12**:2495–2502 (1993).
11. A. Okitani, M. Matsuishi, T. Matsumoto, E. Kamoshida, M. Sato, U. Matsukura, M. Watanabe, H. Kato, and M. Fujimaki. *Eur. J. Biochem.* **171**:377–381 (1988).