Determination of Molecular Mobility of Lyophilized Bovine Serum Albumin and γ-Globulin by Solid-State ^{1}H NMR and Relation to Aggregation-Susceptibility

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Purpose. Feasibility of solid-state ^{1}H NMR for determining the mobility of protein molecules in lyophilized cakes was considered. The mobility in cakes with various levels of water content was studied in relation to aggregation-susceptibility.

Methods. Spin-spin relaxation time (T_2) of protons in lyophilized bovine serum albumin (BSA) and γ-globulin (BGG) was measured as a function of hydration level by solid state ^{1}H NMR using a 'solid-echo' pulse sequence. Moisture-induced aggregation of the lyophilized proteins was also determined by high performance size exclusion chromatography.

Results. Lyophilized BSA and BGG became susceptive to aggregation when water content exceeded about 0.2 g/g of protein. T_2 of protein protons in the lyophilized cakes started to increase at lower water contents. The increase in aggregation susceptibility observed with increasing water content appears to follow the increase in T_2 of protein protons. For lyophilized BGG, both aggregation and T_2 of protein protons decreased at water contents above 0.5 g/g protein.

Conclusions. Mobility of protein molecules in lyophilized cakes was successfully detemined by solid-state ^{1}H NMR. The aggregation susceptibility of proteins was strongly related to their molecular mobility as indicated by T_2.

KEY WORDS: molecular mobility; spin-spin relaxation time; aggregation; solid-echo.

INTRODUCTION

The chemical and physical stability of solid pharmaceutical proteins is largely affected by moisture content (1,2). It has been demonstrated that the aggregation of various lyophilized proteins is enhanced by moisture sorption (3–8). These effects of hydration on the stability of lyophilized proteins appear to be related to the increased molecular mobility of solid proteins.

Increased molecular mobility of solid proteins upon hydration has been detected by various methods. The intramolecular dynamics of freeze-dried myoglobin and trypsin as measured by Mossbauer spectroscopy increased with increasing levels of hydration (9). Hydration-induced flexibility of lysozyme and chymotrypsin was detected by dielectric relaxation studies (10). The internal mobility of lysozyme molecules as measured by hydrogen exchange rates increased strongly with increasing hydration, and was not coupled with the surface motion as indicated by the motion of a spin probe noncovalently bound at the protein surface (11).

Although solid-state NMR is an established method for measuring molecular mobility, few studies have employed this method in order to determine the molecular motions of pharmaceutical proteins in lyophilized formulations, and to elucidate their dependence on water content. In this study, the spin-spin relaxation time (T_2) of protons in lyophilized bovine serum albumin (BSA) and γ-globulin (BGG) was measured by solid state ^{1}H NMR as a function of hydration level. Moisture-induced aggregation of the lyophilized proteins was also determined by high performance size exclusion chromatography. The effect of water content on the protein aggregation is discussed with respect to the mobility of protein molecules as measured by T_2.

MATERIALS AND METHODS

Materials

BSA (essentially fatty acid free, A7511) and BGG (G5009) were purchased from Sigma Chemical Co. (St Lousi, MO) and used without further purification. Deuterium oxide (>99.75%) was purchased from Wako Pure Chemical Industries, Ltd (Osaka).

Preparation of Lyophilized Proteins

BSA and BGG were dissolved in distilled water or deuterium oxide at a protein concentration of 10%w/w. Polypropylene sample tubes (10 mm diam.) containing 200 μl of protein solution were immersed in liquid nitrogen for 10 min, and the frozen samples were dried at a vacuum level less than 5 Pa for 23.5 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo). The shelf temperature was controlled between −35 and −30°C for the first 1 h-period, and at 30°C for the last 3.5 h-period. The shelf temperature during the intermediate period was ambient.

The lyophilized samples were stored at 15°C and 23.4% relative humidity (RH) (adjusted with potasium acetate-saturated H_2O or D_2O solution), 60.2 %RH (NaBr-saturated solution), 75 %RH (NaCl-saturated solution), 86 %RH (KCl-saturated solution), or 98 %RH (K_2SO_4-saturated solution) for 24 h. Samples with a higher water content were prepared by storage at 15°C and 100 %RH for 2, 4, and 6 days. Water content was determined by the Karl Fisher method (684 KF Coulometer, Switzerland).

^{1}H NMR Measurement

Measurements were carried out at 25 MHz using a pulse NMR spectrometer (JNM-MU25, JEOL, Tokyo). Lyophilized protein samples with various levels of water contents were transferred to a glass tube (8 mm diam.) which was then tightly stoppered and loaded in a NMR sample tube (10 mm diam.). Temperature was kept at 20 and 50°C for BSA, and at 20 and 60°C for BGG. Free induction decay (FID) of protons in protein molecules was obtained immediately after 5 min-equilibrium at these temperatures so that aggregation during measurement may be negligible. The 90° pulses were 2 μs in duration. The 'solid echo', with an echo delay of 10 μs, was used in the detection stage of all measurements, in order to overcome the effects of the dead-time (12). Measurement was repeated 16

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times with a recycling time of 3 s. Integrated FID was used to calculate the spin-spin relaxation time (T_2) of the protein protons.

T_2 of protein protons was calculated from FID signals between 2.6 and 100μs by non-linear least-squares regression analysis according to an equation representing the summation of the Gaussian equation ($\exp(-(t/T_2)^2)$) and Lorentzian equation ($\exp(-t/T_2)$). For lyophilized proteins with a relatively high water content (larger than 0.1 g/g protein), the T_2 of H_2O was calculated according to the Lorentzian equation, then the T_2 of protein protons was calculated by inserting the T_2 of H_2O into the Gaussian-Lorentzian equation. The FID signals used for the calculation of T_2 of H_2O were between 100 and 700 μs for lyophilized proteins with a water content of about 0.16–0.18 g/g protein, and between 400 and 1000 μs for lyophilized proteins with a higher water content.

Determination of Protein Aggregation by High Performance Size Exclusion Chromatography

Lyophilized samples of BSA and BGG with various levels of water content were stored at 50 and 60°C, respectively, for 5 h in tightly stoppered sample tubes. Then, 2 ml of phosphate buffer (pH 6.2, 200 mM) was added to each sample and stored at room temperature for 15 h with occasional gentle stirring. The supernatant of the precipitated proteins was drawn off, diluted with phosphate buffer (pH 6.2, 200 mM) to 300 μg/ml, and injected after filtration (0.45 μm) through a 20-μl loop to a column (Tosoh G3000SW, 30 cm × 7.5 mm, Tokyo) maintained at 30°C. The mobile phase was a 200 mM phosphate buffer (pH 6.2) delivered at a rate of 1 ml/min. The column eluate was monitored at 230 nm (UV detector, Model L4000, Hitachi Ltd., Tokyo).

RESULTS AND DISCUSSION

Mobility of Protein Molecules

The mobility of protein molecules in lyophilized BSA and BGG was successfully determined in terms of the T_2 of protein protons. As reported previously, the mobility of water molecules in polymer-water systems can be assessed in terms of the spin-lattice relaxation time (T_1) of water. The T_1 of $H_2{}^{17}O$ in polymer-water systems such as a poly(vinylpyrrolidone)-water system (13,14) and the β-galactosidase-water system (4) has been determined by the inversion recovery method of ^{17}O-NMR. On the other hand, it is difficult to obtain the T_1 of polymer molecules in the solid state using ordinary inversion recovery because of very small T_2 values. The development of the 'solid-echo' pulse sequence has made it possible to determine the T_1 as well as T_2 of solid polymers (12,15). Mobility of polymer molecules in the solid state can be better represented by T_2 rather than T_1, since the linear relationship between correlation time (a basic parameter for molecular mobility) and the reciprocal of T_1 is uncertain for solid molecules with low mobility. In the present study, the mobility of protein molecules in lyophilized BSA and BGG was studied in terms of the T_2 of protein protons determined by the 'solid-echo' method. T_2 of protein protons was calculated from on-resonance FID signals. Typical FID signals obtained from lyophilized BSA samples with various levels of water content prepared using H_2O and D_2O are shown in Figs. 1A and 1B, respectively. The FID of protons present in the lyophilized BSA and BGG prepared using H_2O (Fig. 1A) was comprised of a Gaussian decay due to protein protons and a Lorentzian decay due to H_2O protons. In contrast, the lyophilized proteins prepared using D_2O (Fig. 1B) exhibited mainly a Gaussian FID due to protein protons. T_2 values calculated for the protein protons are shown in Fig. 2.

The T_2 of protein protons in the lyophilized cakes prepared using H_2O and D_2O increased with increasing water content for both BSA and BGG. For the lyophilized BGG the increase in T_2 became smaller at a water content above 0.4 g/g at 60°C.

The T_2 of protein protons in the lyophilized cakes prepared with D_2O was larger than the T_2 in those prepared with H_2O in the range of water content studied. When proteins are lyophilized from D_2O solution and stored under humidity conditions controlled by salt-saturated D_2O solutions, exchangeable protons in protein molecules (such as −NH and −OH) are replaced by deuterium. Therefore, the FID signals observed from protein protons in lyophilized cakes prepared with D_2O are assigned mainly to non-exchangeable protons. Since −NH are found primarily in the main chains of protein molecules, the T_2 of protein protons observed in lyophilized cakes prepared with D_2O can be considered to represent the mobility of protons mainly in the side chains of protein molecules. Most protons of −NH may exchange during preparation of the lyophilized cakes (namely, during processes using proteins in solutions and freeze-drying process), although exchange of −NH protons inside protein molecules is slower than those on molecular surface.

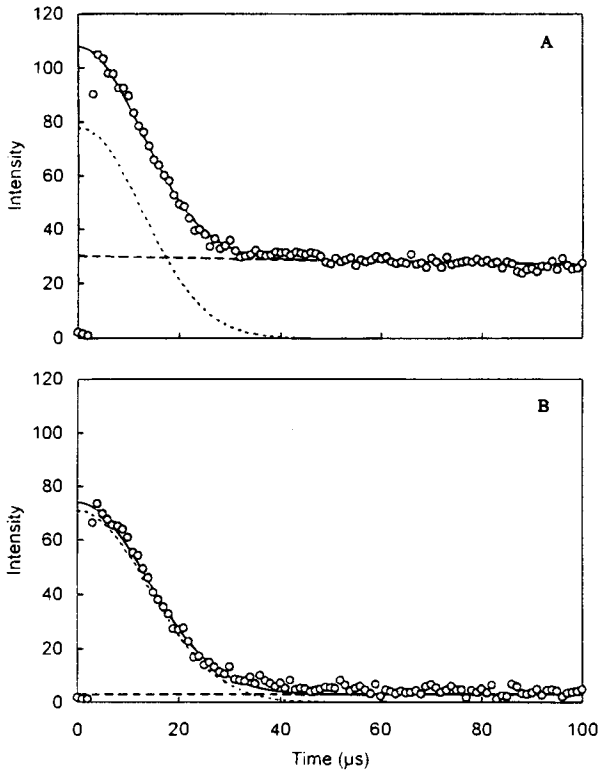

Fig. 1. On-resonance FID signals obtained from lyophilized BSA with water contents of 0.233 and 0.252 g/g protein prepared using H_2O (A) and D_2O (B), respectively, at 20°C. The decay was analyzed into Gaussian (- - - -) and Lorentzian decay (— — —).

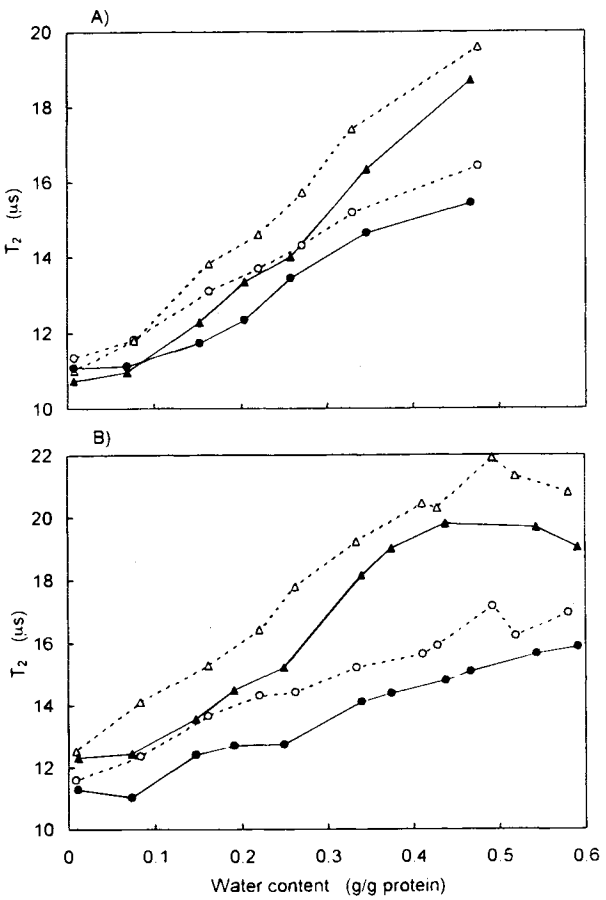

Fig. 2. Spin-spin relaxation time of protein protons in lyophilized BSA (A) and BGG (B) prepared with H_2O (●▲) and D_2O (○△) as a function of water content. Measurement was carried out at 20 (●○), and 50°C and 60°C for BSA and BGG, respectively (▲△). T_2 represents the average value of three measurements.

The larger T_2 observed in lyophilized cakes prepared with D_2O may indicate that the side chains of protein molecules start to increase its mobility upon less hydration than the main chains do and exhibit higher mobility than the main chains in the range of water content studied. The difference in T_2 between the cakes prepared with D_2O and H_2O was less than 2 μ s. This small difference indicates that FID from exchangeable protons and non-exchangeable protons cannot be resolved separately. This is why the cakes prepared with H_2O, which contained both exchangeable and non-exchangeable protons, provided a single Gaussian FID.

Aggregation Susceptibility of Lyophilized Proteins

BSA and BGG are well known to aggregate during storage at elevated temperature (3,7,8). In the present study, aggregation of BSA and BGG in lyophilized cake during storage at elevated temperature was determined by size exclusion chromatography. Figures 3 and 4 show typical chromatograms of the lyophilized BSA and BGG stored at 50°C and 60°C, respectively. Peak a was assigned to intact protein for both BSA and BGG, since protein solution prior lyophilization exhibited only peak a. Before storage, the lyophilized BSA and BGG yielded peaks representing unfolded and aggregated proteins (b and c) as well

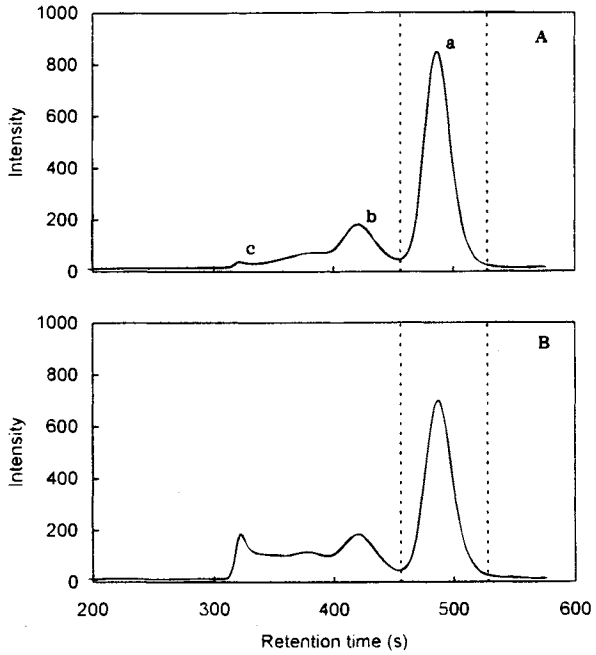

Fig. 3. High performance size exclusion chromatograms of lyophilized BSA with a water content of 0.257 g/g protein before storage (A) and after storage at 50°C for 5 h (B).

as a peak representing intact protein (a). This indicates that BSA and BGG undergo unfolding and aggregation to some extent during freeze-drying. After storage for 5 h at elevated temperature, peak a became smaller and peaks b and c larger, indicating that unfolding and aggregation occurred during storage. The peak area between the two dashed lines, which corre-

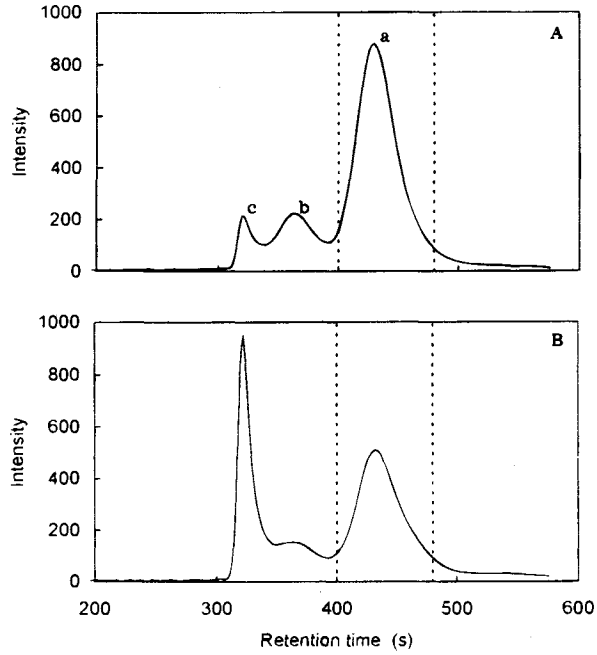

Fig. 4. High performance size exclusion chromatograms of lyophilized BGG with a water content of 0.284 g/g protein before storage (A) and after storage at 60°C for 5 h (B).

sponds to the remaining intact proteins, was calculated and plotted against water content in Fig. 5.

The amount of intact BSA and BGG remaining after storage depended on the water content of the lyophilized cake. Intact protein decreased significantly at a water content above approximately 0.2 g/g protein for both BSA and BGG. BSA exhibited a continuous decrease in remaining proteins as water content increased, within the range of water content studied. BGG exhibited a similar decrease in remaining proteins, but an increase in remaining proteins was observed at higher water contents. The minimum of remaining intact protein was observed at a water content of about 0.5 g/g protein. These results indicate that aggregation via unfolding of the lyophilized proteins is affected by water content in a complex manner.

Molecular Mobility and Aggregation Susceptibility

It is very interesting to compare the water content dependence of aggregation behavior (Fig. 5) with the water content-dependence of the mobility of protein molecules (Fig. 2). Lyophilized BSA and BGG became susceptive to aggregation when water content exceeded about 0.2 g/g of protein. On the other hand, the molecular mobility of BSA and BGG as measured by the T_2 of protein protons started to increase at lower water contents. The increase in aggregation susceptibility appears to follow the increase in protein mobility. Lyophilized BSA exhibited continuous increases in protein mobility and in aggregation susceptibility with increasing water content up to 0.5 g/g protein at 50°C. For lyophilized BGG, the increase in protein mobility became smaller (mobility even appeared to decrease) at a water content above 0.4 g/g protein at 60°C. Aggregation of BGG decreased with increasing water content in this range of water content. These results suggest that the aggregation susceptibility of proteins is strongly related to the molecular mobility of proteins.

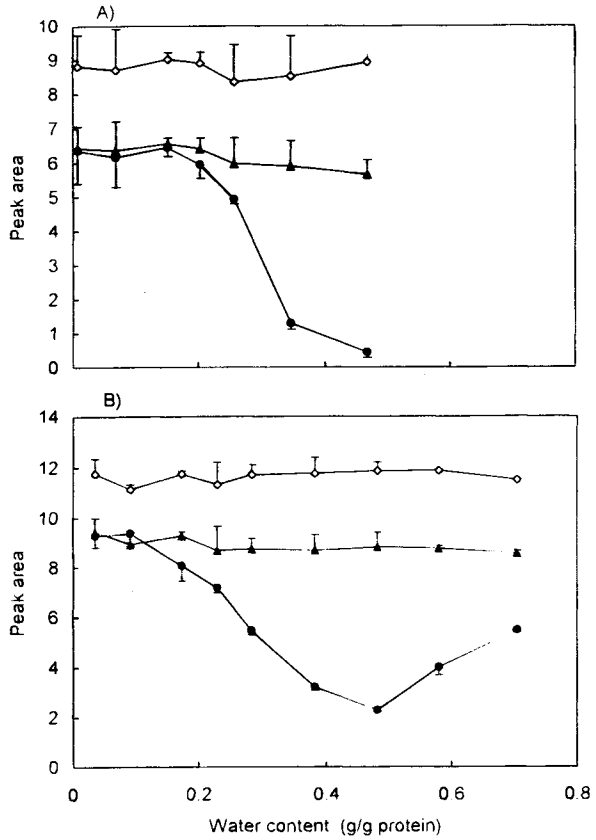

Fig. 5. The effect of water content on aggregation of lyophilized BSA (A) and BGG (B). Peak area of peak a representing intact BSA determined before storage (▲) and after storage at 50°C and 60°C for BSA and BGG, respectively, for 5 h (●). Total peak area is presented by (◇). SD (n = 3).

Studies on hydration of solid lysozyme reported that interaction at strong water binding sites of proteins (charged groups) was completed at a water content of 0.07 g/g protein, and that full monolayer coverage was completed at 0.38 g/g protein (11). The relatively small increase in T_2 at water contents lower than 0.1 g/g protein, which was observed in the present study, suggests that protein mobility as indicated by T_2 is increased by water weakly bound to protein molecules, but not by water strongly bound. Protein mobility increases as weakly-bound water increases, resulting in increased aggregation. The constant or decreasing protein mobility observed for lyophilized BGG with a water content larger than 0.4 g/g protein can be explained by assuming that an increase in water content brings about no increase in protein mobility in the range of water content higher than that needed to produce monolayer coverage. The apparent decrease in BGG mobility observed at a water content above 0.5 g/g protein may indicate some changes in the physical state of the lyophilized cakes, which may lead to decreased aggregation. Decreased aggregation at similar water contents was also observed for BSA and human serum albumin, which has been ascribed to "water's critical role in maintaining protein structure" (3,6).

In conclusion, mobility of protein molecules in lyophilized cakes was successfully determined by solid-state ^{1}H NMR. It was indicated that aggregation susceptibility of proteins in lyophilized cakes is strongly related to the molecular mobility of proteins as indicated by T_2.

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