J. Pharm. Pharmacol. 1994, 46: 182–185
Received April 28, 1993

The Aggregation of Bovine Serum Albumin in Solution and in the Solid State

GERALDINE M. JORDAN*, SUMIE YOSHIOKA AND TADAO TERAO

National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

Abstract—The aggregation of bovine serum albumin (BSA) in the solid state and in solution was studied to elucidate the effect of water mobility on the aggregation rate and mechanism. The results suggest that the freeze-dried BSA forms covalently-bonded aggregates via disulphide bonding during storage in the moistened solid state and in solution. The aggregation rate largely depended on the water content. The freeze-dried BSA to which a small amount of water was added (in the moistened state) was found to be more liable to aggregation than that in solution. The aggregation rate in the moistened solid state exhibited a maximum at a very low level of moisture. In contrast, the aggregation rate in solution increased with increasing ratio of water to protein (with decreasing protein concentration). The results suggest that the increased rate is related to the increase in water mobility, as measured by the spin-lattice relaxation time, T_1, of water using ^{17}O NMR, with increasing ratio of water to protein.

Aggregation has often been observed during storage of various proteins for pharmaceutical use. It has been demonstrated that protein aggregation involves noncovalent processes such as hydrophobic interaction (Pikal et al 1991) or covalent processes due to disulphide-bond interchange (Ahern & Klibanov 1985; Volkin & Klibanov 1987; Liu et al 1991) and other chemical interactions (Townsend & DeLuca 1988, 1991; Hora et al 1992). It appears that proteins form aggregates in a manner which depends on the chemical and physical states of the proteins.

In previous papers we have reported that freeze-dried β-galactosidase with low moisture content aggregated via covalent disulphide bonding, while the aggregation of β-galactosidase in aqueous solution involved noncovalent bonding (Yoshioka et al 1993b). The aggregation rate was found to depend largely on the water content (Yoshioka et al 1993a). It has been demonstrated that the aggregation of lyophilized proteins in the solid state is affected by the water content (Townsend & DeLuca 1990; Yoshii et al 1990; Kitabatake et al 1990; Liu et al 1991). Although water undoubtedly plays an important role in protein aggregation, the mechanism of the participation is still unclear. We have determined the mobility of water molecules in freeze-dried β-galactosidase containing various amounts of water, as measured by the spin-lattice relaxation time, T_1, and suggested that the aggregation rate is related to the mobility of water molecules.

In the present work, we have studied the aggregation of another model protein, bovine serum albumin (BSA), in the solid state and in solution. This study aims at elucidating the effect of water mobility on the aggregation rate and mechanism.

Materials and Methods

Materials

Bovine serum albumin (essentially fatty acid free, A7511) was purchased from Sigma Chemical Co. (St Louis, MO). Two impurities of larger molecular size were found in native polyacrylamide gel electrophoresis.

Bovine serum albumin (BSA) was dissolved in 0·1 M NaCl at a protein concentration of 50 mg mL^{-1} and dialysed at 4°C in 0·1 M NaCl. The pH of the solution was determined to be 5·2. The dialysed BSA solution was used without further purification. Polypropylene sample tubes (14 mm diam.) containing 100 μL BSA solution were immersed in liquid nitrogen for 3 min, and the frozen samples were dried at a vacuum level less than 3 Pa for 15 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo). The shelf temperature was controlled between −40 and −35°C for 14 h, then increased to 35°C. At this temperature drying was continued for 1 h. The freeze-dried samples contained 5 mg BSA and 0·6 mg NaCl in a sample tube. The water content was found to be less than 0·5% by the Karl Fisher method (684 KF Coulometer, Switzerland).

Aggregation of bovine serum albumin

Various amounts (3–5000 mg) distilled water were added to 5·6 mg freeze-dried sample. Samples were stored at 55 or 60°C after being sealed tightly, and removed at appropriate intervals for high-performance size exclusion chromatography measurement. The freeze-dried samples without addition of water were also stored as controls.

For native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) measurements, freeze-dried samples were stored in solution (protein concentration, 5 mg mL^{-1}) at 60°C for 8 h, and in the moistened solid state (water content: 0·54 mg mg^{-1}) at 55°C for 2 h.

High-performance size exclusion chromatography (HPSEC)

The aggregation of the BSA samples stored under various conditions was determined by HPSEC. An adequate amount of 200 mM phosphate-buffered solution (PBS) (pH 6·2) was added to the samples to make a 0·1 mg mL^{-1} protein solution, stirred, and injected through a 20-μL loop to a column (Tosoh G2000SW, 30 cm × 7·5 mm, Tokyo) maintained at 30°C after filtration (0·45 μm). The mobile phase

*Present address: School of Pharmacy, Trinity College, Dublin, Republic of Ireland.
Correspondence: S. Yoshioka, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

was 200 mM phosphate buffer (pH 6·2), which was delivered at a rate of 1 mL min^{-1}. The column eluate was monitored at 230 nm.

Polyacrylamide gel electrophoresis (PAGE)

The BSA samples stored under various conditions were dissolved in 50 mM PBS (pH 7·4), and subjected to PAGE. For SDS-PAGE, the samples were heated at 100°C for 5 min in pH 7·4 PBS containing 2·5% SDS. For SDS-PAGE under reducing conditions, the samples were heated in a similar way, except that the pH 7·4 PBS used contained 5% 2-mercaptoethanol as well as 2·5% SDS. PhastSystem (Pharmacia, Uppsala) was employed for the electrophoresis. PAGE and SDS-PAGE procedures were performed on PhastGel (gradient 4–15) at 15°C at a constant voltage of 400 and 150 V, respectively. Staining was carried out using 0·1% Coomassie brilliant blue R-250 in 30% methanol, 10% acetic acid, as described by Townsend & DeLuca (1991).

^{17}O NMR measurement

^{17}O NMR of the BSA samples containing various amounts of water was determined with a Varian spectrometer (VXR-400S) at 54·2 MHz. The sample tubes were kept at 55°C. Spin-lattice relaxation times, T_1, of $H_2{}^{17}O$ were obtained by using the inversion recovery method. A 90-degree ^{17}O pulse width of 50 μs and a recycling time of 250 ms were used.

Results and Discussion

Fig. 1a shows the HPSEC chromatogram obtained for the freeze-dried BSA before storage. The freeze-dried sample exhibited a peak due to intact BSA at a retention volume of 6·5 mL and a peak due to impurities of higher molecular size at a smaller retention volume. This chromatogram was almost the same as that obtained for the BSA solution before freeze-drying. Fig. 1 shows the chromatograms obtained for the freeze-dried samples stored in the moistened solid state after adding 0·54 mg water (mg freeze-dried solid)$^{-1}$, and for the samples stored in solution after adding 179 mg water (mg freeze-dried solid)$^{-1}$ (protein concentration: 5 mg mL^{-1}). When the sample was stored in solution, the peak due to intact BSA became smaller and a sharp peak appeared at a retention volume near the void volume. This peak is ascribed to soluble aggregates formed during storage. When the sample was stored in the moistened solid state, the formation of the soluble aggregates decreased and the formation of precipitates was observed. It is indicated that BSA tends to form soluble aggregates in solution and insoluble aggregates in the solid state. This is supported by the results of SDS-PAGE and PAGE of these aggregates.

FIG. 1. The HPSEC of the freeze-dried BSA stored in the moistened solid state (water content: 0·54 mg (mg freeze-dried solid)$^{-1}$ at 55°C for 60 min (b), and that stored in solution (protein concentration: 5 mg mL^{-1}) at 60°C for 4 h (c) compared to the HPSEC of the freeze-dried BSA before storage (a).

Fig. 2 shows the results of the SDS-PAGE under non-reducing (lanes a) and reducing conditions (lanes b). Under non-reducing conditions the sample had been heated in an SDS solution containing no reducing agent. Lane 1 shows the results of a control BSA sample that had not been freeze-dried. Lane 2 represents the results of a BSA sample that had been freeze-dried; lane 3, a freeze-dried sample stored in solution (protein concentration: 5 mg mL^{-1}); and lane 4 a freeze-dried sample stored in the moistened solid state (water content: 0·54 mg mg^{-1}).

Some impurities were detected in both SDS-PAGE and reducing SDS-PAGE of the control BSA. Although the SDS-PAGE of the sample stored in solution (lane 3a) had a band at a position which corresponded to a higher mol. wt (arrow 2), no clear band was observed other than the peak due to BSA monomer (arrow 1) in the SDS-PAGE of the sample stored in the moistened solid state (lane 4a). In contrast, no difference was observed in the reducing SDS-PAGE between the samples stored in solution and in the moistened solid state. A clear band due to BSA monomer appeared in reducing SDS-PAGE, even with the sample stored in the moistened solid state (lane 4b) which exhibited only a narrow band due to BSA in the absence of reducing agent (lane 4a). These results indicate that BSA is likely to

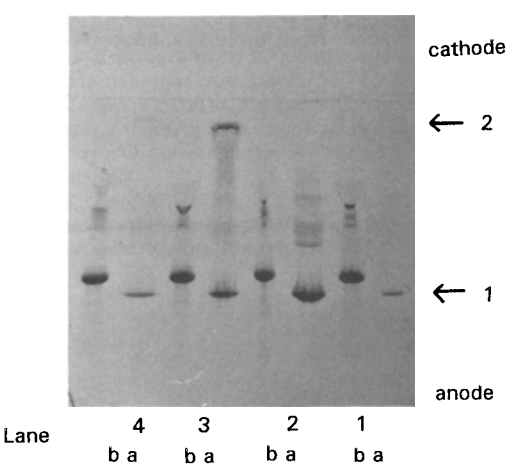

FIG. 2. The SDS-PAGE of the freeze-dried BSA. Lane 1: control BSA sample that had not been freeze-dried; lane 2: BSA sample that had been freeze-dried; lane 3: freeze-dried sample stored in solution (protein concentration: 5 mg mL^{-1}) at 60°C for 8 h; lane 4: freeze-dried sample stored in the moistened solid state (water content: 0·54 mg mg^{-1}) at 55°C for 2 h. a: SDS-PAGE; b: reducing SDS-PAGE.

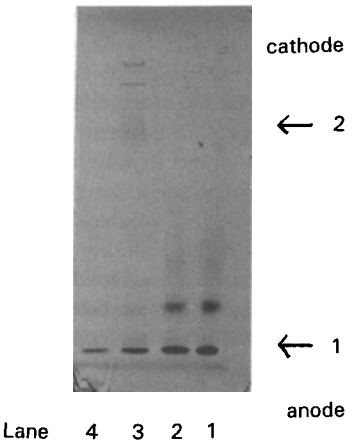

FIG. 3. The native-PAGE of the freeze-dried BSA. Lane 1: control BSA sample that had not been freeze-dried; lane 2: BSA sample that had been freeze-dried; lane 3: freeze-dried sample stored in solution (protein concentration: 5 mg mL^{-1}) at 60°C for 8 h; lane 4: freeze-dried sample stored in the moistened solid state (water content: 0·54 mg mg^{-1}) at 55°C for 2 h.

form soluble aggregates in solution and to form insoluble aggregates (precipitates) in the presence of low moisture. It also indicated that soluble aggregates formed in solution and insoluble aggregates formed in the moistened solid state, both dissociate in the presence of SDS and reducing agent. This suggests that disulphide bonding is involved in the formation of the aggregates. In the SDS-PAGE the band due to BSA monomer at arrow 1 in Fig. 2 shifted to a higher mol. wt position under reducing conditions compared with non-reducing conditions. This suggests that the BSA monomer becomes larger in size when the disulphide bonding of the intact BSA molecule is reduced.

The results of PAGE (Fig. 3) indicate that the aggregation via hydrophobic interaction can be regarded as negligible. The freeze-dried sample stored in solution (lane 3) and the freeze-dried sample stored in the moistened solid state (lane 4) exhibited a band corresponding to intact BSA (arrow 1). This suggests that the peak observed at a position corresponding to BSA monomer in the SDS-PAGE (arrow 1 in Fig. 2) does not result from the dissociation of aggregates

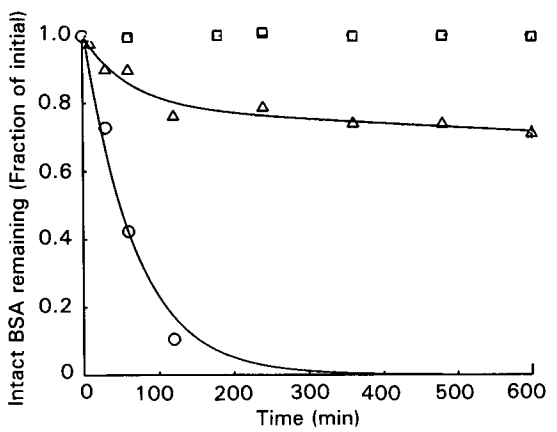

FIG. 4. The time courses of the aggregation of freeze-dried BSA stored in the moistened solid state at 55°C. Water content: 0 (□); 0·54 (○); 0·89 mg (mg of freeze-dried solid)$^{-1}$ (△).

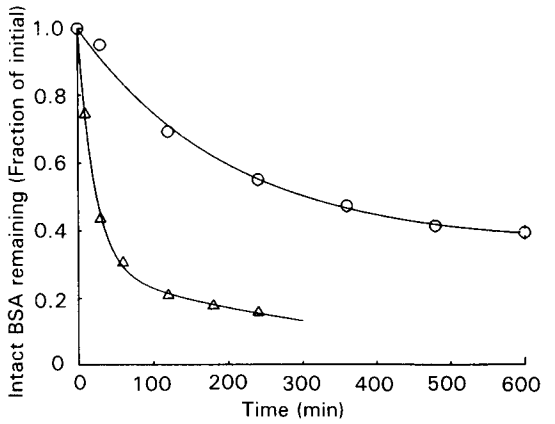

FIG. 5. The time courses of the aggregation of freeze-dried BSA stored in solution at 55°C (○) and 60°C (△). Protein concentration: 5 mg mL^{-1}.

with SDS. The sample stored in solution exhibited another faint band at a position which corresponded to a higher mol. wt in the PAGE analysis (arrow 2), suggesting the formation of soluble aggregates. In contrast, no soluble aggregates were detected for the sample stored in the moistened solid state, even though the band due to intact BSA was faint. This confirms that BSA forms insoluble aggregates in the presence of low moisture.

Fig. 4 shows the time courses of the aggregation of the freeze-dried BSA stored at 55°C in the moistened solid state. The amount of remaining BSA was determined from the peak corresponding to intact BSA observed in the HPSEC, and represented as the ratio of the peak height to the initial peak height. Although no aggregation was observed for the freeze-dried BSA containing no added water, the BSA samples containing 0·54 and 0·89 mg water (mg freeze-dried solid)$^{-1}$ exhibited aggregation at a significant rate. The aggregation rate decreased when the added water increased from 0·54 to 0·89 mg mg^{-1}. Fig. 5 shows the time courses of the aggregation of the freeze-dried BSA stored in solution (protein concentration: 5 mg mL^{-1}) at 55 and 60°C. The aggregation could not be fitted by first-order kinetics since it involved two kinetically distinct processes, i.e. a rapid initial phase followed by a slower second phase. This was the case

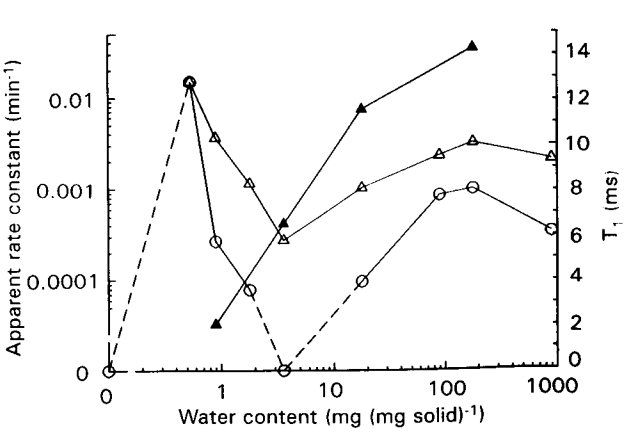

FIG. 6. The apparent rate constant of BSA aggregation at the initial phase (△) and the second phase (○), and the T_1 of $H_2{}^{17}O$ (▲), at 55°C as a function of the amount of water added to freeze-dried BSA.

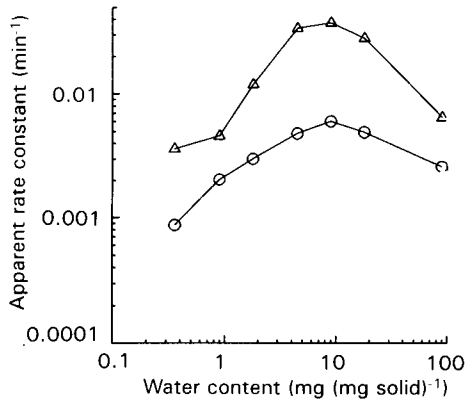

FIG. 7. The apparent rate constant of BSA aggregation at the initial phase (△) and the second phase (○) as a function of the amount of water added to freeze-dried BSA at 60°C.

for the aggregation both in the moistened solid state and in solution. An apparent first-order rate constant was determined for the aggregation in each phase, and regarded as a relative measure of the aggregation rate.

Figs 6 and 7 show the apparent rate constant at 55 and 60°C, respectively, as a function of the amount of water added to the freeze-dried BSA. A minimum aggregation rate was observed for the BSA sample containing 3·6 mg mg^{-1} of water.

The aggregation of the freeze-dried BSA in the moistened solid state seems to exhibit a maximum rate at a very low moisture level. A similar relationship between aggregation rates and water content has been reported for the aggregation of lyophilized BSA containing a few μL of 0·15 M NaCl and 5 mM phosphate buffer (pH 7·3) (Liu et al 1991).

At a water content above 3·6 mg mg^{-1}, on the other hand, the freeze-dried BSA aggregated at an increasing rate with increasing water added, in a similar way to β-galactosidase freeze-dried from phosphate buffer (Yoshioka et al 1993a). The increase in the aggregation rate of β-galactosidase with increasing water content has been explained by the increase in the mobility of water molecules present in the system, as well as by the increase in pH. The increase in the amount of added water brought about an increase in the pH because of the solubility differences of the phosphate species of the buffer (Na_2HPO_4 and KH_2PO_4), resulting in enhanced aggregation of β-galactosidase. The increase in the amount of added water also caused an increase in the mobility of water molecules around proteins, as measured by the T_1 of water, resulting in enhanced aggregation. The BSA samples used in the present study were freeze-dried from 0·1 M NaCl containing no phosphate, thus the pH was about 5·2 over the range of the water content studied. Therefore, no variation in pH is expected from the increase in the water content.

The T_1 of water in the freeze-dried BSA containing various amounts of water is shown in Fig. 6. An increase in the T_1 was observed with increasing water content, suggesting an increase in water mobility. The increase in the aggregation rate of BSA appeared to be related to the increase in the water mobility in a similar way to β-galactosidase.

The increase in the aggregation rate with increasing water content can be ascribed to the increase in water mobility. In contrast, the increase in the rate with decreasing water content, which was observed for the moistened solid state with a very small level of moisture, may be related to differences in salt concentration caused by the difference in the amount of water added to the freeze-dried samples containing NaCl, since salts are known to affect protein stability.

References

Ahern, T. J., Klibanov, A. M. (1985) The mechanism of irreversible enzyme inactivation at 100°C. Science 228: 1280–1284

Hora, M. S., Rana, R. K., Smith, F. W. (1992) Lyophilized formulations of recombinant tumor necrosis factor. Pharm. Res. 9: 33–36

Kitabatake, N., Tahara, M., Doi, E. (1990) Thermal denaturation of soybean protein at low water contents. Agr. Biol. Chem. 54: 2205–2212

Liu, W. R., Langer, R., Klibanov, A. M. (1991) Moisture-induced aggregation of lyophilized proteins in the solid state. Biotech. Bioeng. 37: 177–184

Pikal, M. J., Dellerman, K. M., Roy, M. L., Riggin, R. M. (1991) The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharm. Res. 8: 427–436

Townsend, M. W., DeLuca, P. P. (1988) Use of lyoprotectants in the freeze-drying of a model protein, ribonuclease A. J. Parent. Sci. Tech. 42: 190–198

Townsend, M. W., DeLuca, P. P. (1990) Stability of ribonuclease A in solution and the freeze-dried state. J. Pharm. Sci. 79: 1083–1086

Townsend, M. W., DeLuca, P. P. (1991) Nature of aggregates formed during storage of freeze-dried ribonuclease A. J. Pharm. Sci. 80: 63–66

Volkin, D. B., Klibanov, A. M. (1987) Thermal destruction processes in proteins involving cystine residues. J. Biol. Chem. 262: 2945–2950

Yoshii, H., Furuta, T., Noma, S., Noda, T. (1990) Kinetic analysis of soy-protein denaturation by a temperature-programmed heat denaturation technique. Agr. Chem. 54: 863–869

Yoshioka, S., Aso, Y., Izutsu, K., Terao, T. (1993a) Stability of β-galactosidase, a model protein drug, is related to water mobility as measured by ^{17}O nuclear magnetic resonance (NMR). Pharm. Res. 10: 103–108

Yoshioka, S., Aso, Y., Izutsu, K., Terao, T. (1993b) Aggregates formed during storage of β-galactosidase in solution and in the freeze-dried state. Pharm. Res. 10: 687–691