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Aggregation of Proteins and its Prevention by Carbohydrate Excipients: Albumins and γ-Globulin

MANOHAR KATAKAM AND AJAY K. BANGA

Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn, AL 36849-5503, USA

Abstract

Moisture-induced (2–10 μL added to 10 mg) aggregation of solid-state albumin and γ-globulin was investigated by incubation at 37°C for 24 h.

The insoluble aggregates were centrifuged from a reconstituted solution, dissolved in a solution containing denaturant and reducing agent, and analysed by a Bio-Rad protein assay kit. Of the three albumins used, maximum aggregation (8·2%) was observed with bovine serum albumin that was essentially fatty-acid free. The maximum aggregation observed with γ-globulin was 7·0%. A bell-shaped curve for percent aggregation was observed with increasing moisture content and was especially prominent for bovine serum albumin. When mixed with carbohydrate excipients in a 1:1 ratio, aggregation was reduced for both bovine serum albumin and γ-globulin by all four of the following excipients used: Emdex, dextrose, trehalose and hydroxypropyl β-cyclodextrin. For bovine serum albumin, the aggregation was reduced about sixfold, with Emdex being the most effective excipient.

The likely mechanism of the resulting aggregation was covalent linkages formed due to intermolecular thiol disulphide interchange.

Aggregation is a major concern for the formulation and delivery of peptide and protein drugs. It may lead to immunogenic reactions due to an increase in molecular weight, blockage of tubing, membranes or pumps in infusion sets, reduced bioactivity, and unacceptable physical characteristics that may give an undesirable appearance. Aggregation may be induced by shaking, heating, lyophilization or adsorption to hydrophobic surfaces. γ-Globulin and albumin were selected as model drugs.

Serum albumin, being readily available, is often used as a model protein for physical or chemical studies. Albumin is acidic, highly soluble, and resistant to denaturation. The isoelectric point of albumin containing fatty acids is about 4·8 (Peters 1985). Albumin has a low content of tryptophan and methionine, with only two tryptophan residues in bovine serum albumin and only one in human serum albumin. However, albumin has a high content of cysteine and charged amino acids. The high total charge aids its solubility while the many disulphide bonds contribute to its stability. The ellipsoid molecule has a single polypeptide chain that is organized into a series of nine loops maintained by 17 disulphide bridges. The disulphide bridges connect the sulphurs of two cysteine side chains by covalent bonds. Although the *trans* peptide bond does not allow adjacent cysteine residues to form disulphide bridges with each other, they bridge elsewhere in the polypeptide chain and thus help to maintain the loop structure and stability of the molecule.

Tryptophan residues are mainly responsible for the fluorescence of albumins, with an excitation maximum of 295–305 nm and an emission peak at 343 nm. Because the additional tryptophan of bovine albumin is more exposed than the other, the fluorescence intensity ratio of bovine serum albumin to human serum albumin is 2·7 instead of 2·0. Upon unfolding, exposure of the buried tryptophan residue of the albumin molecule gives higher fluorescence. This provides one of the best available tools to study denaturation.

γ-Globulins comprise a heterogeneous family of proteins with antibody activity and constitute about 20% of total plasma proteins. There are five classes of immunoglobulins (Ig), designated IgG, IgA, IgM, IgD, and IgE. Of these, IgG constitutes about 75% of the total serum immunoglobulins (Goodman 1991).

IgG has shown a highly pronounced tendency to aggregate when heated to 60°C at neutral pH (Singh et al 1991). This heat-induced aggregation reverses itself upon cooling and this reversibility indicates that non-covalent forces are involved. On the other hand, shaking-induced aggregation of monoclonal antibodies results in precipitation, presumably due to unfolding at the air/water interface followed by aggregation (Levine et al 1991). The study by Levine et al (1991) suggested that surface-induced denaturation can be prevented by surfactants such as polysorbate 80 which preferentially adsorb at the surface. Another study (Burgess et al 1992) suggested methodology to measure interfacial shear rheology of proteins, which can be used to predict competition between a surfactant molecule and IgG for interfacial adsorption.

γ-Globulin aggregated in phosphate buffer, pH 7·2, by adsorbing to the hydrophobic interphase (Thurow & Geisen 1984). This was prevented by addition of polypropylene glycol/polyethylene glycol block polymer. Aggregation was prevented by Genapol PF-80, apparently because it contains long polyethylene glycol chains that prevented the adsorption of dissolved proteins at hydrophobic interfaces.

Correspondence: A. K. Banga, Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn, AL 36849-5503, USA.

Materials and Methods

Materials

Bovine serum albumin (essentially fatty-acid free; moisture content 3·7%), human serum albumin (essentially fatty-acid free; moisture content 6·7%), human serum albumin (crystallized and lyophilized; moisture content 4·5%), human γ-globulin (from Cohn fraction II, III; moisture content 1·0%), sodium azide, and trehalose, were obtained from Sigma Chemical Company (St Louis, MO, USA). Sodium phosphate monobasic, sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, propylene glycol, dithiothreitol, urea, disodium ethylenediamine tetraacetate (EDTA), and dextrose, were obtained from Fisher Scientific Company (Fairlawn, NJ, USA). Emdex was a gift from Mendell, Inc. (Patterson, NY, USA) and hydroxypropyl-β-cyclodextrin (HP-β-CyD) was a gift from Wacker Chemie GmbH (Munich, Germany). Bio-Rad protein assay kit was obtained from Biorad Laboratories (Richmond, CA, USA). The instruments used included an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silverspring, MD, USA), a Dynac II Centrifuge (Clay Adams, Franklin Lakes, NJ), a Vertis shell freezer (Virtis Research Equipment, Gardiner, NY) and a Labconco freeze drier (Labconco Corporation, Kansas City, MO, USA).

Experimental methods

Buffers. All buffers were prepared immediately before use with triple-distilled water and filtered through 0·22 μm filters. The pH adjustments used 0·1 M sodium hydroxide or 0·1 M hydrochloric acid solutions. Most studies were at pH 7·4 ± 0·02 using phosphate buffer (ionic strength 0·1 M) consisting of $9{\cdot}45 \times 10^{-2}$ M dibasic sodium phosphate, $5{\cdot}5 \times 10^{-3}$ M monobasic potassium phosphate and 0·015 M sodium chloride. Sodium azide (0·02%) was used as a preservative.

Preparation of protein samples for moisture-induced aggregation. The preparation technique described here was used to characterize the insoluble aggregates of albumin and γ-globulin. Four types of proteins, γ-globulin, human serum albumin, human serum albumin essentially fatty-acid free, and bovine serum albumin essentially fatty-acid free were used. Protein (10 mg) was transferred to a 5-mL test-tube. Two, three, five, seven, or ten microlitres of moisture in the form of phosphate buffer was added to the test-tubes. Samples were thoroughly mixed using a stainless steel spatula, taking care to remove the wet protein sticking to the spatula and returning it to the sample tube. The tubes were sealed with Parafilm and covered with aluminium foil. These were stored in a stability oven at 37 ± 2°C for 24 h. The protein was reconstituted with 10 mL phosphate buffer and vortexed for 5 min. After standing for 30 min, the tubes were centrifuged for 20 min at 2500 rev min^{-1}. This generated an insoluble pellet of aggregated samples at the bottom of the tube. The aggregates were solubilized in a reducing solution consisting of 1 mM EDTA and 10 mM dithiothreitol in phosphate buffer. All experiments were conducted in triplicate.

Bio-Rad protein assay. Standard solutions of 0·2, 0·4, 0·6, 0·8, and 1 mg mL^{-1} protein were prepared in phosphate buffer. Unknown samples were prepared by dissolving the aggregated albumin pellets in the reducing solution and 100 μL from each of the standard and sample solutions was placed in a 10-mL test-tube with 5 mL Bio-Rad solution. After 10 min, solutions were analysed spectrophotometrically at 595 nm. A plot of the absorbances of these standard solutions was prepared as explained by the supplier, which was used to determine the corresponding protein content.

Preparation of samples for the excipient study. Moisture-induced aggregation of bovine serum albumin and γ-globulin aggregation was studied in the presence of dextrose, Emdex, HP-β-CyD and trehalose. Ten milligrams of protein was placed in a 5-mL glass test-tube to which 10 mg of one of the excipients was added and mixed thoroughly with a steel spatula. To this mixture, moisture as phosphate buffer was added and mixed thoroughly with a spatula. The quantity of moisture used was 5 μL for bovine serum albumin and 3 μL for γ-globulin. Care was taken to remove and return any wet protein that stuck to the spatula. These samples was sealed with Parafilm, covered with aluminium foil and stored at 37°C for 24 h. All experiments were conducted in triplicate.

Fluorimetric analysis. Rectangular quartz fluorescence cells with a path length of 10 mm were used for all fluorescence measurements. These cells were soaked in 50% nitric acid for approximately 30 min and rinsed with triple-distilled water and dried. For particular experiments, the same cell was used to minimize variability that might be attributed to the cell. The excitation and emission wavelengths were 283 and 351 nm, respectively. Phosphate buffer was used as the blank. Solutions of aggregated bovine serum albumin were measured for fluorescence intensity. Readings were in arbitrary fluorescence units, although comparisons against standard solutions were made when required.

Lyophilization-induced aggregation. Bovine serum albumin (10 mg) was dissolved in 10 mL phosphate buffer and transferred to a Labconco flask. The flask was placed in a shell freezer with a −40°C methanol bath to freeze the solution as a thin film inside the whole surface area of the flask. The flask was then connected to the vacuum valve of the freeze-drier and allowed to lyophilize over 24 h. The lyophilized samples were reconstituted with 10 mL buffer and analysed by fluorescence. Lyophilization was also induced in the presence of excipients.

Results and Discussion

Moisture-induced aggregation in the solid state

The level of moisture needed to induce maximum aggregation was investigated. This study used bovine serum albumin (essentially fatty-acid free), human serum albumin (essentially fatty-acid free), human serum albumin (lyophilized and crystallized), and γ-globulin. The results are shown in Figs 1, 2. The γ-globulin used had a 99%

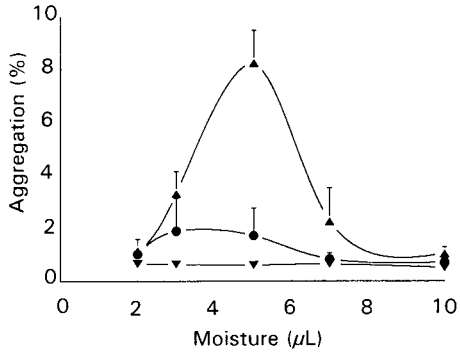

FIG. 1. Moisture-induced aggregation of serum albumins in the solid state. ▲ Bovine serum albumin (fatty-acid free), ● human serum albumin (lyophilized), ▼ human serum albumin (fatty-acid free).

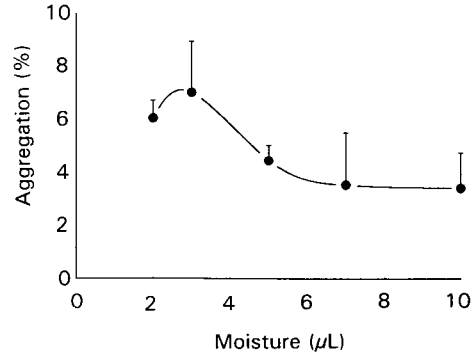

FIG. 2. Moisture-induced aggregation of γ-globulin in the solid state.

electrophoretic purity, as specified by the supplier, and contained IgG as its primary constituent.

As the moisture increased from 2 to 10 μL, a bell-shaped curve was observed for aggregation of bovine and human serum albumin. The maximum aggregation (8·2%) was observed for bovine serum albumin with 5 μL moisture (Fig. 1). Of the three albumins investigated, the most pronounced bell-shaped curve was found for bovine serum albumin, which also exhibited the highest aggregation. Thus, all further studies were conducted with bovine serum albumin to study the stabilizing potential of formulation excipients. For γ-globulin (Fig. 2), the maximum aggregation (7%) was observed with 3 μL moisture. It should be noted that the moisture added was in addition to the 1·0–6·7% water content of the proteins used.

Although some soluble aggregates might have been generated, the Bio-Rad assay would not distinguish these from the nonaggregated protein. Thus, only the insoluble aggregates were measured. The insoluble aggregates, which formed a pellet at the bottom of the test-tube, failed to dissolve in water even with vigorous stirring. Using the technique of Liu et al (1991), these aggregates were dissolved in a solution of 6 M urea, 10 mM dithiothreitol and 1 mM EDTA. The dithiothreitol reduced the intermolecular disulphide covalent linkages between albumin molecules, whereas urea loosens the aggregate making all covalent disulphide bonds accessible to the thiol reagent and thereby solubilizing the aggregates. A chelating agent, EDTA, was used to prevent auto-oxidation. This mechanism of solubilization suggested that aggregates involved covalent bonds.

Two factors, moisture and incubation at 37°C, contributed to aggregation and most likely had a synergistic effect. Bovine serum albumin has a free thiol of a cysteine residue which can undergo interactions with disulphide bridges of other albumin molecules. Hydration levels at or below the monolayer level are insufficient for mobilization of reactants (Hageman 1988). Monolayer coverage of bovine serum albumin needs about 8–10% moisture, whereas the particular lot of bovine serum albumin used in the present study had a reported water content of only 3·7%. This might even explain why the control, to which no moisture was added, did not aggregate even when incubated at 37°C. Beyond monolayer coverage, the loosely bound water is available to mobilize the reactants since the effective viscosity of bound water decreases to allow easier diffusion of reactants. The reaction rate will increase to a point where all the reactants have been solubilized. However, at still higher levels of moisture the dilution of the reactants acts to inhibit the reaction. The temperature required for thermal denaturation of proteins decreases with increasing hydration until the water content reaches 50% (Hageman 1988). In the present study, 5 μL moisture with bovine serum albumin and 3 μL with γ-globulin may have increased water content above monolayer formation, but not so much to dilute the reactants, thus resulting in maximum aggregation at this level. At this level, moisture and temperature act synergistically to denature the protein. Below this level, the hydration may be less than monolayer coverage, while above this level the reactants may be solubilized and diluted resulting in a lowered tendency towards aggregation. The net result is that aggregation shows a maximum, or a peak, at just above the monolayer formation, thus producing a bell-shaped curve as seen in Figs 1, 2.

Fluorescence study of aggregates

Fluorescence of the aromatic amino acid tryptophan was used as an indicator to investigate the mechanism of moisture-induced aggregation. Tryptophan, being hydrophobic, normally lies in the interior of the protein molecule. Upon unfolding, the protein molecule exposes the inner hydrophobic moieties. These moieties tend to self associate, which leads to aggregation. Therefore, the extent of aggregation can be determined by the increased fluorescence of the aggregated samples.

After diluting all the aggregated samples to equal concentrations their fluorescence was measured. The samples with higher aggregation exhibited higher fluorescence (Fig. 3). This suggested that higher aggregation, as indicated by higher precipitation, also involves greater covalent interactions. These interactions are preceded by the unfolding of the polypeptide chain exposing the inner hydrophobic tryptophan moiety and resulting in higher fluorescence.

Lyophilization-induced aggregation

Lyophilization increased the aggregation of bovine serum albumin, as measured by its fluorescence intensity (Table 1). During the freezing of the albumin solution in phosphate buffer, ice crystals of pure water form, causing a localized increase in the salt concentration of the interstitial unfrozen liquid, which could induce aggregation of the protein. This aggregation was reduced by Emdex and dextrose as

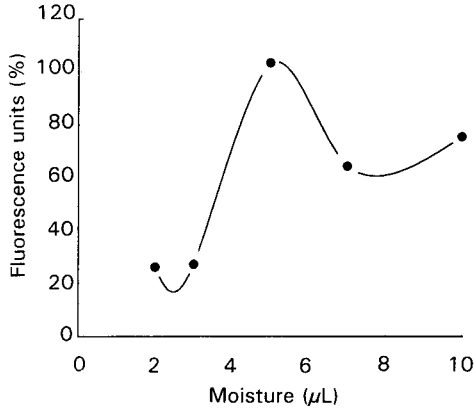

FIG. 3. Fluorescence of bovine serum albumin aggregated with different moisture levels.

excipients, to a level below that of the untreated (control) sample.

Use of excipients to prevent moisture-induced aggregation

Four carbohydrate excipients, Emdex, dextrose, HP-β-CyD, and trehalose, were investigated at a 1:1 ratio of protein to excipient for their potential to prevent aggregation. Since protein drugs are very potent, a reasonable amount of excipient is often needed to provide the bulk to facilitate processing. For bovine serum albumin, Emdex was the most effective excipient to minimize aggregation, followed by HP-β-CyD, dextrose and trehalose (Table 2). For γ-globulins, dextrose and Emdex were equally effective, followed by HP-β-CyD and trehalose. The mechanisms by which excipients stabilize proteins against aggregation or other decomposition reactions are not well understood. A vast number of compounds have been investigated for their ability to stabilize proteins. Creighton (1983) stated, 'the variety of effects observed is quite staggering... the net effect is often a subtle interplay among effects on both the folded and unfolded states of a protein'. In general, the protein structure is stabilized by a combination of hydrogen bonding, electrostatic interactions, hydrophobic interactions, cross-linking and binding of metal ions, with hydrophobic interactions being considered the single most important factor in stabilizing the three-dimensional structure. The effect of excipients on protein stability may be discussed based on their effects on these various forces and interactions.

Stabilizing effects of carbohydrates on proteins have been explained based on their influence on the structure of water which determines the strength of hydrophobic interactions. Arakawa et al (1991) hypothesized that proteins in the solid state were stabilized by sugars which act as an active water substitute to satisfy the hydrogen bonding. Hydrophobic interactions are stronger in the presence of sugars than in pure water (Back et al 1979). Also, since sugars have a lower dielectric constant than water, the electrostatic interactions are likely to be stronger.

Emdex is known as dextrates and complies with the official monograph in NF XVII as the hydrated form. It contains 93–99% of dextrose and 2–5% of maltose. Emdex has good flow and compression characteristics and is used as a tableting excipient. Emdex exists almost entirely as free-flowing spray-crystallized porous spheres. These physical attributes might allow Emdex to better hold moisture, and increase its effectiveness in reducing aggregation. HP-β-CyD is a cyclic oligosaccharide with a ring structure that resembles a truncated core. It also has a hydrophobic inner cavity that can make inclusion complexes. The hydrophobic side chains of aromatic amino acids of proteins may be entrapped in the hydrophobic cavity of HP-β-CyD, hence protecting the protein from adsorption and aggregation (Matsuyama et al 1987; Simpkins 1991).

Table 1. Lyophilization-induced aggregation of bovine serum albumin and its prevention by excipients.

	Fluorescence intensity
Control	39 ± 3·0
Lyophilized	49 ± 0·0
Emdex	21 ± 6·0
Dextrose	30 ± 1·5

Values are mean ± s.d.

Table 2. Effect of carbohydrate excipients on the moisture-induced aggregation of bovine serum albumin and γ-globulin in the solid state.

	Amount aggregated (%)
Bovine serum albumin	
Control	6·0 ± 1·7
Dextrose	1·2 ± 0·5
Emdex	0·7 ± 0·0
HP-β-CyD	0·8 ± 0·2
Trehalose	1·3 ± 0·1
γ-Globulin	
Control	9·6 ± 1·1
Dextrose	4·1 ± 1·0
Emdex	4·5 ± 0·4
HP-β-CyD	5·8 ± 0·4
Trehalose	7·5 ± 0·3

Values are mean ± s.d.

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