

HEAT DENATURATION OF HUMAN SERUM ALBUMIN. MIGRATION OF BOUND FATTY ACIDS

JOHNNY BRANDT and LARS-OLOV ANDERSSON

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

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Prolonged heat treatment of solutions of human serum albumin at 60°C resulted in formation of one aggregate fraction and one fraction that was stable against further heat treatment. Fatty acid analyses on these fractions indicate that the heat stable fraction is formed by migration of fatty acids from the aggregating molecules to the remaining monomer thereby stabilizing the latter against heat denaturation. Disulphide and SH groups are involved in the aggregation process.

A property which earlier was thought to be a general characteristic of proteins was their tendency to precipitate or coagulate when heated in solution. This phenomenon attracted the interest of many investigators in the early days of protein chemistry. However, despite the great number of studies on the heat denaturation of proteins there remain a number of unsolved questions.

One of the proteins which has been extensively investigated with regard to heat denaturation is serum albumin. The kinetics of the process has been studied and it has been observed that heat treatment causes serum albumin to behave as if it was a heterogeneous population of molecules. This has been taken as support for the concept of the heterogeneity of albumin (1,2). The present study provides a possible explanation of the heterogeneous behaviour of albumin following heat denaturation, and also some data on the aggregation process.

MATERIALS AND METHODS

Lyophilized human serum albumin (Rdp 254) prepared by ethanol fractionation was obtained from AB KABI, Stockholm. It contained about 7% dimer, and polymer and monomeric albumin was prepared by gel filtration on a Sephadex G-150 column (3.2 × 100 cm). The column was eluted

with 0.1 M Tris-HCl buffer pH 7.5 containing 0.5 mM EDTA. The molecular weight of the albumin monomer was assumed to be 68,000. The fatty acid content of the monomer fraction was 1.42 moles of long-chain fatty acids per mole albumin and the SH content was 0.38 moles SH per mole albumin. Defatted albumin was prepared by treatment with charcoal according to Chen (3). Before use the charcoal was allowed to settle three times in distilled water to remove fine particles.

Determination of the amount of fatty acids bound to albumin was performed using gas chromatography as described by Hagenfeldt (4). A known amount of albumin was extracted with an acid mixture of isopropanol and heptane. The unesterified fatty acids were then extracted from the heptane phase with alkaline methanol. The methanol phase was then acidified and the fatty acids were extracted into heptane. The heptane extract was concentrated and the fatty acids were methylated with diazomethane according to Schlenk & Gellerman (5). The individual fatty acids were then determined by gas chromatography using heptadecaonic acid as internal standard.

The SH content of serum albumin was determined by disulphide exchange reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) essentially as described by Janatova et al. (6). A stock solution of 0.01 M DTNB in 0.05 M phosphate buffer

pH 7.0 was prepared. The reaction mixture was then prepared by mixing 0.8 ml of the stock DTNB solution and 0.2 ml of a 0.025 M EDTA solution pH 7.0 with 3.0 ml of albumin solution pH 7.0. The absorbance of the reaction mixture was read against a blank solution at 412 nm after the solutions had been allowed to stand for 45 min at room temperature. The concentration of albumin in the reaction mixture was in the range 1–3%. Corrections were made for light scattering effects and the SH content was then calculated using a molar extinction coefficient of $13600 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Heating of albumin solutions, 5% w/v protein in 0.05 M sodium phosphate buffer pH 7.0, was carried out in closed glass tubes in a thermostated water bath. The temperature was controlled to $\pm 0.2^\circ\text{C}$. The amount of aggregates formed in the samples was determined by gel filtration on a Sephadex G-150 column ($2.0 \times 80 \text{ cm}$) in 0.1 M Tris-HCl buffer pH 7.5 containing 0.5 mM EDTA. The absorption of the eluate at 280 nm was determined, and corrections made for light scattering effects.

RESULTS

A 5% solution of monomeric albumin in 0.05 M phosphate buffer pH 7.0 was heated to 60°C . Aliquots of the solution were removed at various time intervals and the degree of aggregation and the SH content of the samples were determined. The percentage of aggregates and the content of SH groups in the unfractionated samples were plotted against time. The results obtained are shown in Fig. 1. It is obvious that aggregates form rapidly and that the SH content decreases at the beginning of the heating period, but that a con-

stant value is reached after about 1 h, both with respect to SH content and aggregates. The aggregation proceeded initially by formation of a small amount of dimer followed by a large formation of higher polymers. The content of dimer was not more than 5% of total protein at any stage of the reaction.

The monomer and polymer fractions obtained after heating for 10 h were subjected to fatty acid analysis. The values obtained were 2.56 moles fatty acids per mole albumin for the monomer fraction and 0.12 moles fatty acids per mole albumin for the polymer fraction. The polymer fraction is thus almost devoid of bound fatty acids, while the content in the monomer fraction is much higher than in the starting monomeric material.

The role of fatty acids in the aggregation process was further investigated by adding fatty acids to the albumin solutions before heating. A 5% solution of monomeric albumin was heated to 60°C for 2 h at pH 7.0 in the presence of 3 mM potassium palmitate. Aggregates could not be detected in this sample by gel filtration. However, a 5% solution of defatted albumin heated to 60°C was very unstable and formed a gel in about 1 h. The presence of fatty acids evidently stabilizes the albumin against heat denaturation.

It has previously been indicated that SH groups and disulphide bonds play an important role in heat denaturing processes (7,8). Some experiments were performed on samples of albumin in which the SH groups were blocked before heating. An albumin solution with blocked SH groups was prepared by treatment with excess N-ethylmaleimide (NEMI) (9). To a 1% solution of monomeric serum albumin in 0.1 M phosphate buffer pH 7.0, was added an equal volume of a 10^{-3} M solution of NEMI in 0.1 M phosphate buffer pH 7.0. The

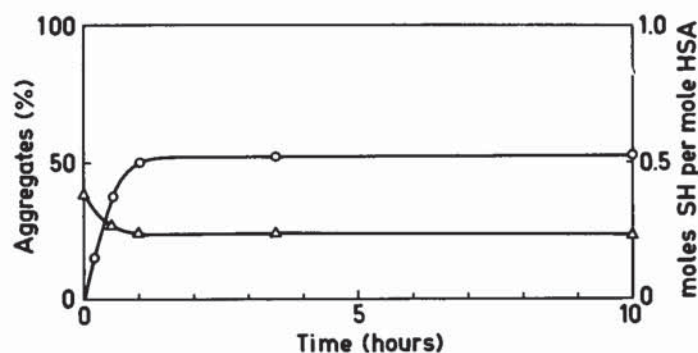


FIGURE 1

Kinetics for the aggregation of a 5% solution of monomeric human serum albumin on heating at 60°C in 0.05 M sodium phosphate buffer pH 7.0.

—○— percentage of aggregates, —△— content of SH in the unfractionated samples.

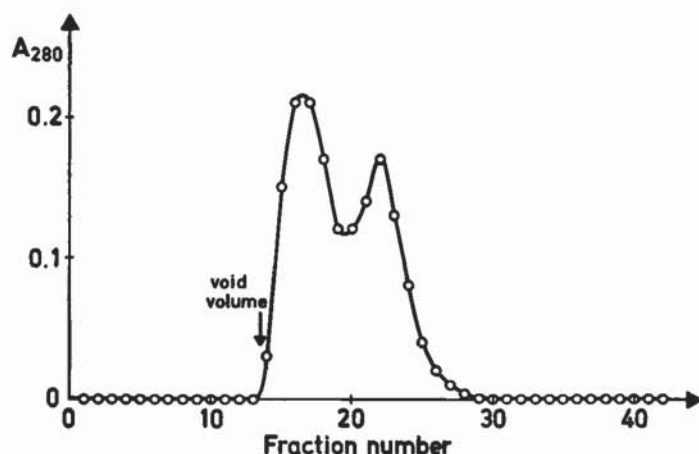


FIGURE 2

Elution diagram from a Sepharose 6 B gel filtration of 10 mg albumin polymer that had been reduced and alkylated with iodoacetamide. The elution was performed in 7 M urea, 0.1 M ammonia-ammonium acetate buffer pH 9.2.

mixture was stored at room temperature for 3 h and the excess reagent removed by Sephadex G-25 gel filtration. The material appearing at the void volume was concentrated by ultrafiltration. Determination of the SH content of this sample showed a value of less than 0.01 moles SH/mole albumin. A 5% solution of this albumin sample was stable to heating at 60°C for 10 h. No aggregate formation could be detected in the sample by gel filtration on Sephadex G-150.

In a second experiment, an albumin solution with blocked SH groups was prepared by treatment with cystine according to King & Spencer (10). A 1% solution of monomeric albumin in 0.1 M Tris-HCl buffer pH 8.0 in the presence of 10^{-3} M L-cystine was allowed to stand at room temperature for 24 h. Excess reagent was removed by Sephadex G-25 gel filtration and the material appearing at the void volume was concentrated by ultrafiltration. The SH content of this sample was less than 0.01 mole SH/mole albumin. A 5% solution of this albumin sample, however, was not stable to heating at 60°C. The time course of aggregation was about the same as that obtained with an untreated sample. The manner of blocking the SH groups is evidently of great importance.

Some experiments were also performed to investigate the nature of the bonds providing aggregate stability. The protein from the polymer fraction obtained after gel filtration of a 5% solution of monomeric albumin that had been heated for 10 h at 60°C, was reduced and alkylated essen-

tially as described by Crestfield et al. (11) except that iodoacetamide was used as the alkylating agent instead of iodoacetic acid. About 10 mg of the polymer fraction was reduced with 0.3 M 2-mercaptoethanol in 8 M urea at pH 8.6 for 4 h and the sample was then treated with a slight excess of iodoacetamide. The sample was subjected to gel filtration on Sepharose 6 B in 7 M urea, 0.1 M ammonia-ammonium acetate buffer pH 9.2. The elution curve is shown in Fig. 2. The material appearing in the latter peak showed the same elution volume as the native albumin that had been reduced and alkylated as described above. It was thus evident that only about 40% of the polymer fraction could be converted to monomer by this treatment.

In another experiment the polymer fraction was reduced and alkylated as described above except that 6 M guanidinium-HCl was used as the denaturing agent instead of urea. Gel filtration of this sample on Sepharose 6 B in 7 M urea showed the same elution pattern as in Fig. 2.

Oxidation of the polymer fraction with performic acid according to Hirs (12) and subsequent gel filtration on Sepharose 6 B as described above also gave an elution curve equal to that shown in Fig. 2. It is thus very probable that most of the aggregates formed during the heat treatment are held together by covalent bonds that are not disulphide bonds.

DISCUSSION

The kinetics of the heat aggregation of serum al-

bumin shows that after some time no more aggregates are formed. This result agrees with the results of earlier studies on the heat denaturation of serum albumin (1,13) showing that at a given temperature a heat-stable fraction is formed which is resistant to further heat treatment. It has been proposed that this phenomenon may be related to microheterogeneity in the secondary and tertiary structure of serum albumin (1,2) and it has been shown that it could not be explained by heterogeneity due to the occurrence of mercapt- and nonmercaptalbumin (2).

It is evident from earlier studies that albumin can be stabilized against heat denaturation by fatty acid anions (14). These results are confirmed in this study and it is also shown that the stable fraction formed after heat treatment has an increased content of fatty acids and that the aggregated fraction is almost free from fatty acids. There are two explanations for this. One is that there is migration of bound fatty acids from unfolding albumin molecules to remaining albumin molecules thereby stabilizing the latter to unfolding. The other possibility is that the fatty acid distribution is initially uneven, due for instance, to heterogeneity in the fatty acid binding ability of the albumin molecules. However, isoelectric focusing and pH-solubility experiments (15–17) give some information about the fatty acid distribution in various albumin samples. Although there is some heterogeneity in distribution, the results do not indicate that the distribution is as uneven as that obtained by the heat denaturing process. Furthermore, it was recently shown that there is a fairly rapid exchange of the fatty acid molecules bound to albumin (18). This strongly supports the first possibility. The most likely process, however, is a combination of both explanations, namely that albumin molecules containing somewhat lower amounts of bound fatty acids, unfold and aggregate first and thereby lose their fatty acids to other albumin molecules. Unfolding then continues until the residual non-aggregated albumin contains enough fatty acids to be stable against heat treatment at that temperature.

During heat treatment there is a decrease in the SH content concomitant with the aggregation. When the aggregation stops there is no further change in the SH content. The SH groups thus seem to be involved in the aggregation process. This is strongly supported by the complete in-

hibition of aggregation by blocking the SH groups with N-ethylmaleimide.

Disulphide exchange reactions were earlier thought to be of importance in the formation of aggregates, and the data obtained in this study agree in part with this. The failure of the cystine treatment to produce an albumin which is stable against heat probably depends on the trace amounts of SH which are sufficient to allow disulphide exchange reactions to take place. The treatment with N-ethylmaleimide does not leave any SH residues. However, the observation that a considerable fraction of the aggregates are held together by covalent bonds other than disulphide bonds indicates, that although SH groups may be involved in the aggregation process, the links do not necessarily need to be disulphide bonds. Another possibility is that lanthionine is formed and that the link is a thioether bridge. In this case SH groups and disulphide bonds could participate, but the link would not be a disulphide bond. This process was suggested earlier by Jaenicke (19) to occur on heat denaturation at alkaline pH values but perhaps it may also be effective at the neutral pH used in this study.

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Address:

Johnny Brandt

Institute of Biochemistry

University of Uppsala, Box 531

S-75121 Uppsala

Sweden