Possible Role of Denatured Albumin in Formation of "Heat-resistant" Serum Albumin

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(Received May 8, 1980)

Studies with the probe dye 2-(4-hydroxyphenylazo)benzoic acid, which can detect small conformational differences in serum albumin, showed that the conformation of "heat-resistant" serum albumin (form N') was not constant, but depended on the heating temperature, becoming similar to that of the denatured monomer as the heating temperature was increased from 55 °C to 70 °C in buffer of pH 7.0. Denatured albumin was suggested to protect native serum albumin from full denaturation. In the presence of denatured albumin, native albumin was found to undergo only a slight conformational change to give the N' form. This protective effect may explain why a constant amount of serum albumin remains as "heat-resistant" serum albumin even after prolonged heating.

From studies on changes in the solubility and sedimentation velocity of serum albumin on denaturation, Levy and Warner[1,2] concluded that a certain amount of serum albumin remains undenatured even after prolonged heating at 65 °C in solutions of various pH values, and that denatured albumin consists of monomer, dimer and higher aggregates. These findings have been confirmed by electrophoresis of albumin heat denatured between 65 °C and 75 °C.[3-5] The presence of an undenatured, heat-stable fraction in heat-treated albumin has been interpreted as due to the existence of "heat-resistant" serum albumin, and it has sometimes been taken as support for the concept of the heterogeneity of serum albumin.[6,7]

In studies using probe dyes, such as 2-(4-hydroxyphenylazo)benzoic acid (HABA), Bromcresol Green and 8-anilinonaphthalene-1-sulfonate,[8-10] we found that there are at least two forms, N and N', in the native fraction of heat-treated serum albumin, and that during heating N' is formed from N by slight conformational modification. The N' form is "heat-resistant" albumin itself. Although "heat-resistant" albumin (N' form) is very similar to N in properties, such as its helical content, electrophoretical mobility and absorption spectrum in the ultraviolet region,[5] it can be distinguished from N with these probe dyes. Among the probe dyes examined, HABA is the most useful, since it shows a new spectral band at about 480 nm with N or N', but not with denatured albumin.[8,11] The affinity of HABA for N', determined from the absorbance of the dye at about 480 nm, was found to be about 60% of that of N in heat-treated albumin at 65 °C in buffer of pH 7.0 or pH 9.0.[8]

Since heat denaturation of serum albumin is characterized by the presence of "heat-resistant" serum albumin and the formation of aggregated albumin, it is very important for understanding the mechanism of heat denaturation of albumin to determine various properties of these molecular species, and especially of "heat-resistant" albumin. This paper deals with the effect of the heating temperature of the properties of "heat-resistant" serum albumin, and the effect of denatured albumin on formation of "heat-resistant" albumin, examined by electrophoresis and with the probe dye HABA. The importance of denatured albumin in formation of "heat-resistant" albumin is suggested.

Experimental

Materials. Bovine serum albumin (Armour, crystalline, Lot no. H72803 and H72009) was used without further purification. Its molecular weight was assumed to be 6.6×10^4. The concentration of albumin in solution in 0.1 mol/dm^3 potassium phosphate buffer, prepared just before use, was determined spectrophotometrically as described previously.[8] The concentration of denatured albumin was expressed in terms of monomer. HABA was obtained from Wako Chemical Co., Osaka (Japan). The other reagents used were standard commercial products.

Method. Heat denaturation was carried out at pH 7.0 as described previously.[8] Acrylamide gel electrophoresis (gel concentration: 7.5%) was conducted at pH 8.3, and quantitative analysis of protein bands of heat-treated albumin, stained with Amidoblack 10B, was carried out in a Shimadzu dual-wavelength TLC scanner, model CS-900, using a wavelength pair of 590 and 700 nm.[10] The absorption spectrum of HABA was recorded at pH 7.0 and 25 °C in a Union spectrophotometer, model SM 4012.

Results

Effect of Temperature on Properties of N'. Figure 1 shows the time-course of heat-denaturation of 1.5×10^{-4} mol/dm^3 bovine serum albumin at various temperatures at pH 7.0, determined by acrylamide gel electrophoresis. At all temperatures, denaturation was first rapid and then slowed down, finally reaching a steady level. The existence of an undenatured fraction of serum albumin after prolonged heating is regarded as due to the formation of modified native albumin N' ("heat-resistant" albumin) from native albumin.[8] The results in Fig. 1 indicate that the amount of N' decreases as the heating temperature increases; at 55 °C, 92% of native albumin (N) changes to N', whereas at 70 °C, only 20% changes to N' and 80% is denatured. At 80 °C all the native albumin is denatured (Table 1). This temperature-dependent denaturation is essentially the same as that at pH 8.9.[4] However, denaturation proceeded more rapidly at pH 8.9, probably because the exchange of disulfide bonds associated with denaturation of serum albumin was greater due to the higher concentration of OH^- (Refs. 5 and 12).

As reported previously, the spectrum of the probe dye HABA shows a new spectral band at about 480

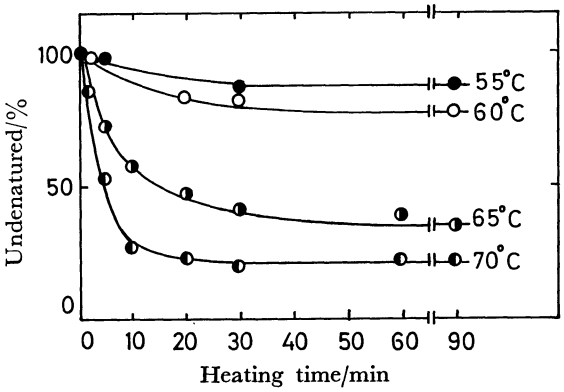

Fig. 1. Time course of heat denaturation of serum albumin at various temperatures determined by acrylamide gel electrophoresis.

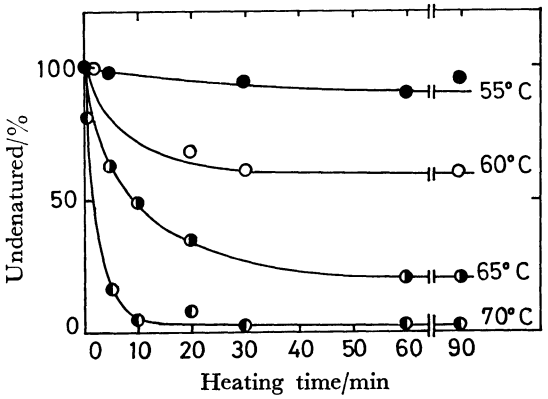

Fig. 2. Time course of heat denaturation of serum albumin at various temperatures determined from the absorbance of HABA.

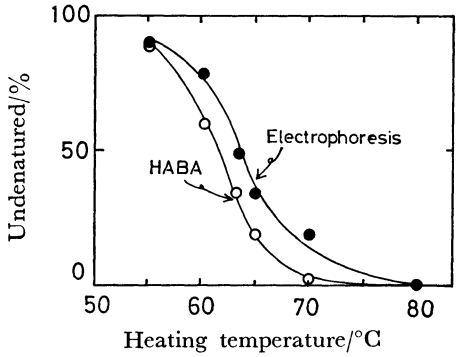

Fig. 3. Temperature-dependence of change in amount of undenatured albumin after heating for 90 min, determined by electrophoresis and from the absorbance of HABA.

nm on binding with undenatured albumin (N and N').[8] Using the same heat-treated albumin as for the experiments in Fig. 1, the absorbance of 10^{-3} mol/dm^3 HABA at 480 nm was measured. The relative absorbance of HABA induced by 10^{-5} mol/dm^3 heat-treated albumin to that induced by the same concentration of unheated albumin was taken as the relative amount of undenatured albumin remaining during heat-treatment, and is plotted as a function of the heating period at various temperatures in Fig. 2. The results in Fig. 2 are very similar to those in Fig. 1 determined by acrylamide gel electrophoresis, but the changes are more rapid.

Table 1. Relative amount of N' formed after prolonged heating

Heating temp/°C	Relative amount of N'/%		Ratio (A/B)
	From absorbance of HABA (A)	By electrophoresis (B)	
55	89	92	0.97
60	60	79	0.76
63	35	50	0.70
65	20	35	0.57
70	3	20	0.15
80	0	0	—

Figure 3 shows the temperature dependences of the relative amount of N' after heating for 90 min, determined by electrophoresis and from the absorbance of HABA. These values and the ratio of the relative amount of N' determined with HABA to that determined by electrophoresis are listed in Table 1. Values for the relative amount of N' determined by the two methods decreased similarly with the heating temperature, but the value determined with HABA was always smaller than that determined by electrophoresis, because the molar extinction coefficient of HABA induced by N' is smaller than that induced by N.[8] If the conformation of N' around the binding sites for HABA is the same in N' formed at all temperatures, the ratio of N' determined with HABA to that determined by electrophoresis should be the same between 55 °C and 70 °C, even though the relative amount of N' determined by the two methods changes with the heating temperature. However, the results in Table 1 show that this ratio becomes smaller with increase in the heating temperature, indicating that the structure of N' is not the same at different heating temperatures, and that the affinity of N' to HABA becomes smaller as the denaturating temperature increases.

Effect of Denatured Albumin on Denaturation. Figure 4 shows the time course of denaturation of serum albumin at 65 °C monitored by measuring the absorbance of HABA at 480 nm; the relative absorbance of HABA induced by heat-treated albumin to that induced by unheated albumin is plotted against the heating time. In this experiment, solutions containing various amounts of denatured albumin (final concentration, D_0) and native albumin (final concentration, N_0) at a constant total protein concentration of 5.0×10^{-5} mol/dm^3 were heated. The denatured albumin used was obtained by heating serum albumin at 80 °C for 15 min in buffer of pH 7.0 to denature it completely. It is apparent from the figure that the extent of denaturation decreases with increase in the amount of denatured albumin; with solutions containing 75% and 50% $D_0(N_0/(N_0+D_0)=$ 0.25 and 0.50, respectively) before heating, the absorbance of HABA due to the undenatured fraction ($N+N'$) after heating for 25 min is decreased to 43%

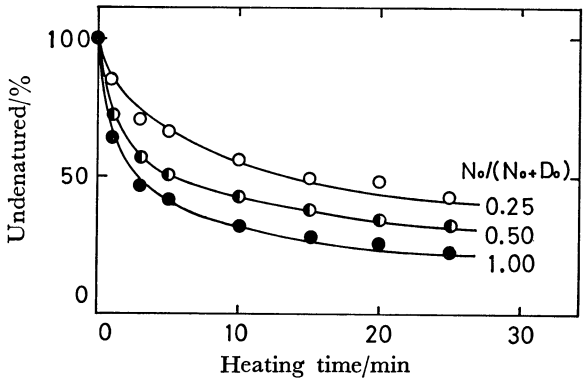

Fig. 4. Time course of heat denaturation of serum albumin at 65 °C and pH 7.0 in the presence of various amounts of heat denatured albumin. Denaturation was monitored by the absorbance of 10^{-4} mol/dm^3 HABA.
N_0: Initial concentration of native albumin, D_0: initial concentration of heat-denatured albumin.

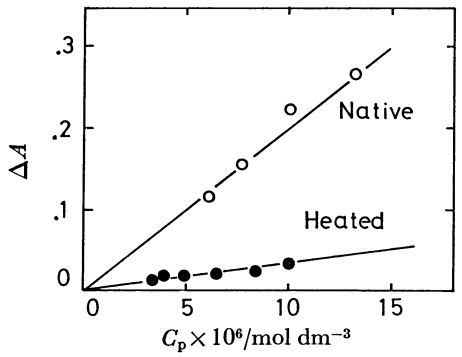

Fig. 5. Concentration dependence of heat denaturation of serum albumin at 65 °C and pH 7.0. The denaturation was monitored as the absorbance of 5.0×10^{-4} mol/dm^3 HABA induced by 10-fold diluted samples of heat-treated albumin. The concentrations of heat-denatured albumin(C_p) shown in the figure are those at the time of measuring the absorbance of HABA, and the C_p values during heat-denaturation were 10 times greater. The absorbance of HABA induced by native albumin is also shown.

and 32%, respectively, while in the absence of denatured albumin ($N_0/(N_0+D_0)=1$) it is decreased to 23%. The addition of denatured albumin before heating seems to inhibit denaturation. However, in this experiment the decrease in denaturation could be due to an effect of concentration on denaturation of serum albumin, since the experiments were carried out keeping N_0+D_0 constant and thus N_0 decreased with increase in D_0.

For examination of this possibility, we measured the heat denaturation at 65 °C of solutions of serum albumin at concentrations of 3.0×10^{-5} mol/dm^3 to 10^{-4} mol/dm^3. The solutions were heated for 100 min, and then mixed with a solution of HABA and the absorbance of HABA was measured at 480 nm. The absorbance induced by various concentrations of native albumin was also measured. As shown in Fig. 5, the absorbance of HABA increased linearly with increase in concentration of the heat-treated albumin,

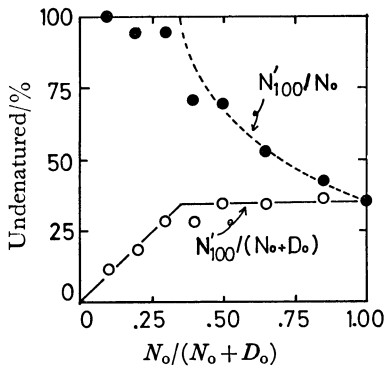

Fig. 6. Effect of heat-denatured albumin on denaturation of serum albumin at 65 °C and pH 7.0.
D_0: Initial concentration of heat-denatured albumin, N_0: initial concentration of native albumin, N'_{100}: concentration of N' after heating for 100 min.

indicating that denaturation does not depend on the concentration of albumin under these experimental conditions. The ratio of the slope of the straight line obtained with denatured albuimn to that with native albumin was 0.18, coinciding well with the value of 0.20 obtained on denaturation of 1.5×10^{-4} mol/dm^3 serum albumin for 90 min at 65 °C (*cf.* Table 1). Thus the decreased denaturation of native albumin in the presence of denatured albumin shown in Fig. 4 could be due to an inhibitory effect of denatured albumin, not to dilution of the native albumin. Thus the effect of denatured albumin on denaturation was next studied in more detail.

Various amounts of native albumin(N) were mixed with denatured albumin(D), obtained by heat-treatment at 80 °C for 10 min, keeping the total albumin concentration at 1.5×10^{-4} mol/dm^3. The mixtures were heated at 60 °C or 65 °C for 100 min, where the albumin remaining undenatured was N' form. Then they were subjected to polyacrylamide gel electrophoresis and the amount of undenatured albumin (N'_{100}) was determined from the absorbance of the stained Amidoblack 10B. The ratio N'_{100}/N_0 (the amount of undenatured albumin as a percentage of that before heating), and the ratio $N'_{100}/(N_0+D_0)$ (the amount of undenatured albumin as a percentage of the total concentration of albumin) are plotted as functions of $N_0/(N_0+D_0)$ (=the relative amount of native albumin before heat-treatment) in Figs. 6 and 7.

On heating at 65 °C (Fig. 6), the value of N'_{100}/N_0 became greater as the value $N_0/(N_0+D_0)$ decreased; in the absence of denatured albumin before heat-treatment ($N_0/(N_0+D_0)=1.00$) N'_{100}/N_0 was 35%, whereas when the albumin solution contained more than 65% denatured albumin ($N_0/(N_0+D_0)\leq0.35$) N'_{100}/N_0 was almost 100%. However, when the percentage of undenatured albumin was expressed in terms of the total albumin content, $N'_{100}/(N_0+D_0)$, 35% of the albumin consistently remained undenatured in the region where the content of the original native albumin was greater than 35% ($N_0/(N_0+D_0)\geq0.35$). Moreover, in the region where $N_0/(N_0+D_0)$ was less than 0.35, the plot of change in the percentage of undenatured albumin after heating for 100 min

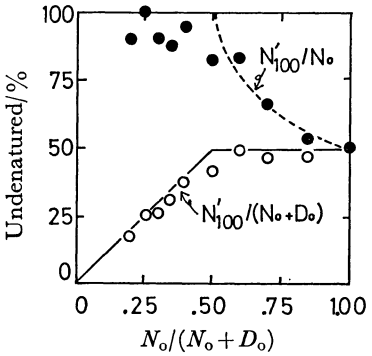

Fig. 7. Effect of heat-denatured albumin on the denaturation of serum albumin at 63 °C and pH 7.0. Experimental conditions were similar to those in Fig. 6, except heating temperature.

against $N_0/(N_0+D_0)$ was linear with a slope of one; that is, the percentage of undenatured albumin was the same as before heat-treatment, since no denaturation took place. In the figure, the solid and dotted lines were drawn assuming that at less than 0.35 $N_0/(N_0+D_0)$, the serum albumin was not denatured.

On denaturation at 63 °C, results were similar to those on denaturation at 65 °C, as shown in Fig. 7, and about 50% of the albumin was denatured when no denatured albumin was added before heat-treatment (the solid and dotted lines in the figure were drawn assuming that at less than 0.50 $N_0/(N_0+D_0)$, the serum albumin was not denatured.). The scatter of experimental results on denaturation at both temperatures in the region of small values of $N_0/(N_0+D_0)$ is due to the difficulty in determining the exact amount of undenatured albumin, since before heating the sample contained only a small amount of native albumin.

Discussion

The results of Figs. 1 and 2, and Table 1 show that form N' ("heat-resistant" serum albumin) remaining in heat-treated albumin after prolonged heating changes in conformation and loses affinity for HABA with increase in the heating temperature. Under the experimental conditions for Fig. 2, where the concentration of the probe dye HABA was much greater than that of the protein, the change in affinity of the protein for the dye reflects change in the number of binding sites for the dye.[8,10] Thus decrease in the affinity of N' for HABA with increase in heating temperature from 55 °C to 70 °C indicates that the number of binding sites on N' became smaller as the heating temperature increased. Since the denatured monomer did not have any binding sites for HABA that induced the characteristic absorption band of the dye, it can be concluded that the conformation of N' became similar to that of the denatured monomer, at least with regard to the microenvironment around the binding site for HABA, with increase in the heating temperature. As reported previously,[9] heat-treatment of native albumin(N) first causes formation of N' and denatured monomer. There must be some regulatory effect determining the amounts of denatured monomer and N' formed from N. Thus the mechanism of formation of N' from N is important for understanding the mechanism of heat-denaturation of serum albumin.

The results of this study, especially those in Figs. 6 and 7, indicate that denatured albumin has a protective effect on heat-denaturation of serum albumin, and that the denaturation proceeds until the ratio of the amount of native albumin to denatured albumin attains a certain value depending on the heating temperature; at 65 °C, 35% was native and 65% denatured, whereas at 63 °C, 50% was native and 50% denatured. Kratochvil *et al.*[13] reported that albumin that had been heated at 65 °C retarded the aggregation of serum albumin induced by heating at 75 °C. This report and our results suggest that denatured albumin stabilizes native albumin, preventing the latter from complete denaturation.

Some biopolymers are known to be stable in solution containing protein or starch.[14] Recently, Martinek *et al.*[15] reported that α-chymotrypsin and trypsin became stable against thermo-inactivation, when they interacted non-covalently with polyacrylamide gel. Similarly, our preliminary experiments showed that the presence of polyvinylpyrrolidone reduced the rate of heat-denaturation of serum albumin. In view of these findings, it is suggested that denatured albumin (D) which mainly consists of aggregated albumin protects native albumin(N) from heat-denaturation probably by a certain non-covalent interaction between D and N, but this "interaction" is not strong enough to prevent the denaturation completely and thus N changes slightly in conformation to form N'. With increase in the heating temperature, the "interaction" between D and N will become weaker, and thus the conformational change of N' will increase, the conformation of N' finally becoming similar to that of the denatured monomer, as indicated by the results in Table 1.

Though it is not clear at present how denatured albumin protects native albumin from denaturation, the stabilizing effect of denatured albumin could be one of the decisive reasons why serum albumin is not completely denatured and "heat-resistant" serum albumin (form N') exists between 55 °C and 70 °C. Since our recent results[10] have suggested that fatty acids also influence the process of heat-denaturation of serum albumin, further studies are necessary on the mechanism of formation of N' during heat-denaturation of serum albumin.

The authors thank Miss Yukari Maeda for technical assistance. The present work was partially supported by a Grant-in-Aid for Scientific Research No. 167273 from the Ministry of Education, Science and Culture.

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