Physicochemical Characterization of Heat-induced Soluble Aggregates of Bovine Globin

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Received September 6, 1982

Physicochemical properties of heat-induced soluble aggregates of bovine globin were studied with several physicochemical measurements and electron microscopy.

A great change in ellipticity and formation of soluble aggregates of globin occurred between 45 and 50°C.

Soluble aggregates of globin formed below 60°C were globular particles with a diameter of 15 to 20 nm and a sedimentation coefficient and molecular weight of approx. 20S and 8×10^5 , respectively. Their solutions were not so highly viscous. On the other hand, soluble aggregates of globin formed above 80°C were fibrous macro-aggregates with a thickness of 8 to 10 nm of which the sedimentation coefficient and molecular weight were above 50S and 10^6 to 10^8 , respectively. Their solutions were highly viscous.

It is suggested that a great increase in globin viscosity on heating takes place through the following process; globular aggregates are formed spontaneously with the unfolding of globin molecules, and subsequently fibrous macro-aggregates are formed *via* extension and association of globular aggregates. It is also considered that fibrous macro-aggregates might form a gel at a higher concentration.

The annual production of blood from slaughtered amounts to about 80 million kilograms in Japan. This blood contains about 18% protein and hemoglobin accounts for more than half of the blood protein. Most of the hemoglobin is not utilized for human consumption at present in Japan because of its disagreeable color and odor.

Bovine globin with a slightly yellow color was prepared from bovine hemoglobin by treatment with acidified acetone,^{1,2)} hydrogen peroxide³⁾ or a proteolytic enzyme.⁴⁾ Recently, Sato *et al.*,⁵⁾ prepared globin with an amino acid composition similar to the original hemoglobin from several kinds of animal blood by using a CM cellulose procedure.

From nutritional studies it is known that the chemical score of bovine globin is very low due to the lack of isoleucine and low methionine content.^{6~10)} However, this does not present

any serious problem in the utilization of globin because its digestibility is extremely high,⁷⁾ and the biological value or protein efficiency ratio can be greatly improved by supplementation with isoleucine and methionine, or cereal protein isolates.^{7~9)}

Successful supplementation to food, and the replacement or simulation of traditional proteinaceous foods will depend on the availability of new proteins having critical functional characteristics which enable them to be used and to be acceptable to people.¹¹⁾ Some functional properties of globin such as solubility,^{1,2,10,12,13)} emulsifying properties^{1,2,10,12)} and foaming properties^{2,10)} have been characterized by several workers. Attempts have been made to incorporate globin into sausage meat,¹⁴⁾ and a cheese-like emulsion was prepared using globin as an ingredient.¹⁵⁾ In the previous work,¹³⁾ it was found that a globin

solution heated above 80°C was highly viscous and formed a transparent gel at the concentration of above 3% under well controlled heat conditions, suggesting that globin forms soluble aggregates on heating. However, little is known as to the properties of soluble aggregates of globin and the process of thermal aggregation and formation of a transparent gel.

The purpose of the present study was to determine the physicochemical characteristics of soluble globin aggregates formed at various temperatures and to discuss the process of gel formation of globin.

MATERIALS AND METHODS

Preparation of bovine globin. Hemoglobin prepared from bovine blood by the method described in the previous study¹³⁾ was further purified by DEAE cellulose column chromatography.¹⁶⁾ Bovine globin was prepared from the purified hemoglobin using the CM cellulose procedure described in the previous article.¹³⁾

Heating conditions. Bovine globin was mainly heated at a concentration of 1% in 0.025 M sodium acetate buffer, pH 5.3, at various temperatures for 10 min in a thermostath controlled within $\pm 1^\circ\text{C}$.

Circular dichroism analysis. Circular dichroism experiments were carried out with a JASCO J-40A spectropolarimeter in a 1 mm cell at 25°C, at a protein concentration of 0.2 mg/ml. The data were expressed in terms of mean residue ellipticity, taking the mean residual weight of globin as 108. Temperature dependence of the circular dichroism spectrum of globin was measured at a constant wavelength of 222 nm, raising the temperature from 25 to 55°C at a rate of 2°C/min. The concentration of the globin solution was 0.1 mg/ml in 0.025 M sodium acetate buffer, pH 5.3.

Measurement of sedimentation coefficient. Sedimentation coefficients were measured with an ultracentrifuge (Hitachi UCA 1A) at 27,160, 43,700 or 60,000 rpm and 25°C in 0.025 M sodium acetate buffer, pH 4 to 5.8, at a globin concentration of 0.5 g/100 ml. All values were normalized to 20°C with pure water as the solvent. The relative ratio of aggregates was calculated from the peak area of the fast sediment component per total peak areas of fast and slow sediment components.

Gel filtration. Gel filtration was performed on a Sephacryl S 200 column (1.6 $\times$ 40 cm) using 0.025 M so-

dium acetate buffer, pH 5.3, for elution buffer. Each eluted fraction (3 ml) was monitored at 280 nm. The relative ratio of aggregates was calculated from the peak area monitored at 280 nm.

Electrophoresis. Electrophoresis was performed on 4.5% flat bed polyacrylamide gel (pH 4.3) containing 0.06 M KOH and 0.378 M acetic acid. The electrophoresis was conducted at 4 mA/cm² and for 6 hr using a running buffer (pH 4.5) consisting of 0.35 M β -alanine and 0.14 M acetic acid. The gel was stained with 0.5% Coomassie brilliant blue R250 and destained with 7% acetic acid containing 10% methanol. The gel was densitometrically monitored with a Shimadzu Dual Wavelength Scanner CS 910. The relative ratio of aggregates was calculated from the peak area of non-aggregated components for heated globin per that for unheated globin. The relative ratio of aggregates was averaged for 3 to 5 runs.

Viscosity measurement. Viscosities of heated globin solutions were measured with an Ostwald viscometer having a flow time of 114 sec for water at 20°C. Reduced viscosity was calculated from relative viscosity based on the concentration of heated globin determined by the method of Lowry *et al.*¹⁷⁾ Intrinsic viscosity was determined by extrapolating reduced viscosity to a concentration of zero.

Density gradient ultracentrifugation. Heated globin (5 mg) was centrifuged in 12 ml of a 5 to 30% (w/v) linear sucrose gradient containing 0.025 M sodium acetate buffer, pH 5.3, in a Hitachi RPS 40 T rotor at 25,000 rpm for 15 hr at 10°C. The gradient was fractionated into 0.6 ml portions with monitoring at 280 nm with an ISCO density gradient fractionator. The protein content of each fraction was determined by the Bio-Rad protein assay method.

Light scattering. Light scattering measurements were performed with a low angle laser light scattering photometer (Chromatix KMX-6, Sunnyvale, USA). The laser was a 2 mW He-Ne unit operating at the red wavelength of 632.8 nm. The scattering angle was 4.5°. All measurements were performed at 20°C. The protein concentration (0.1 ~ 0.2 mg/ml for heated globin and 1 ~ 3 mg/ml for unheated globin) was determined by the method of Lowry *et al.*¹⁷⁾ Refractive increments of unheated and heated globin at 60°C were measured with a laser differential refractometer (Chromatix KMX-16, Sunnyvale, USA) at a wavelength of 633 nm. The molecular weight of heated globin was calculated from the Rayleigh factor and refractive increment of 0.1564 for unheated and heated globin.

Electron microscopy. A Hitachi electron microscope HU 12A was used at an accelerating voltage of 75 kV. A globin solution was diluted 100-fold after heat treatment. A small drop was applied to a carbon-coated copper grid, and 3% uranyl acetate was used for staining.

RESULTS

Figure 1 shows circular dichroism spectra for globin heated at pH 5.3 and various temperatures. Both unheated and heated globin showed minimum ellipticity at around 222 nm and 208 nm. Negative ellipticity of globin at 222 nm greatly decreased on heating between 45 and 50°C. The temperature dependence experiment also showed a great change of ellipticity at 222 nm between 45 and 50°C. Therefore, it was considered that the globin molecules unfolded between 45 and 50°C. Negative ellipticity showed a slight decrease between 60 and 80°C. This decrease may be attributed to the contamination by a small amount of hemoglobin (3~5% in globin preparation) with a melting point value of 67°C.^{18,19)}

Sedimentation coefficients of globin heated at various pHs and temperatures are shown in Table I. The globin solution heated at pH 5.8 was turbid and coagulated above 60°C, whereas that heated at pH 4.0 formed no aggregate. The globin solution heated at pH 5.3 was clear up to 90°C and that heated at 95°C was a gel-like coagulate. Two distinct peaks

were observed when globin was heated at pH 5.3 and 50°C. One was a non-aggregate which disappeared above 60°C, and the other was a

TABLE I. SEDIMENTATION COEFFICIENTS OF GLOBIN HEATED AT VARIOUS pHs AND TEMPERATURES

All values are shown as $S_{20,w}$ at a protein concentration of 5 mg/ml. When heated globin showed two peaks, both sedimentation coefficients are shown in the table, that of the minor peak being in parentheses.

Heating temp. (°C)	pH			
	4.0	4.8	5.3	5.8
30	1.9	2.9	3.7	4.6
40	nd	3.2	4.6	6.0
50	nd	3.5	5.4	24.4
		(14.1)	22.2	31.6
60	nd	3.8	23.1	70.3
		18.2		
70	nd	5.4	29.8	
		21.7		
80	1.7	3.3	52.7	
		33.1		
90	nd	9.7	63.3	
		39.9		
100	2.1	2.6		
		50.2		

nd, not determined.

FIG. 1. Circular Dichroism Spectra of Globin Heated at Various Temperatures.

○, unheated and heated at 40°C; ●, 45°C; △, 50°C; ▲, 55°C and 60°C; □, 80°C.

FIG. 2. Elution Patterns of Gel Filtration on a Sephacryl S 200 for Globin Heated at Various Temperatures.

○, unheated; ●, 50°C; △, 60°C; □, 80°C.

soluble aggregate having a sedimentation coefficient of approx. 20 S. This soluble aggregate showed little increase in sedimentation coefficient but an increase in amount when heated at 60°C. The sedimentation coefficient of heated globin greatly increased above 80°C.

Figure 2 shows the elution pattern of gel filtration for heated globins. Unheated globin showed a single peak corresponding to the non-aggregate and globin heated above 50°C showed an additional peak at a void volume corresponding to the aggregate. The higher the temperature was, the smaller the peak of the non-aggregate.

FIG. 3. Electrophoretic Patterns of Globulin Heated at Various Temperatures.

Heating temperatures are shown in the figure.

FIG. 4. Concentration Dependence of Reduced Viscosity of Globulin Heated at Various Temperatures.

○, unheated; ●, 50°C; △, 60°C; ▲, 70°C; □, 80°C; ■, 90°C; ▽, 60°C and subsequently at 90°C.

Figure 3 shows the electrophoretic patterns of the heated globins. The amount of the fast moving component considered to be a non-aggregate decreased on heating above 45°C, and the amount of the slow moving com-

FIG. 5. Density Gradient Ultracentrifugal Patterns of Globulin Heated at Various Temperatures and Molecular Weights of Their Peak Fractions Estimated by Light Scattering.

○, unheated; ●, 50°C; △, 60°C; □, 70°C; ▲, 80°C; ■, 90°C.

FIG. 6. Mean Residual Ellipticity at 222 nm, Relative Ratio of Aggregates and Intrinsic Viscosity of Globulin Heated at Various Temperatures.

●, ellipticity; ○, relative ratio of aggregates by electrophoresis; □, relative ratio of aggregates by ultracentrifugation; △, relative ratio of aggregates by gel filtration; ▲, intrinsic viscosity.

ponent considered to be a soluble aggregate increased with raising of the temperature to 60°C. Little globin heated above 80°C migrated into the gel.

Figure 4 shows the concentration dependence of reduced viscosities of heated globins. Reduced viscosities of globin heated above 80°C were extremely high, whereas only a slight increase in reduced viscosity was observed on heating below 70°C. Reduced viscosity of globin heated at 90°C showed a high concentration dependence as well as that of that heated at 60°C and then 90°C.

Figure 5 shows the patterns of density gradient ultracentrifugation of heated globins and the molecular weights (MW) of their peak

fractions determined by light scattering. The peak tended to move to a higher density, the higher the temperature was. The MWs of peak fractions of globin heated at 50 to 70°C were around 8×10^5 . The average MW of the whole fraction of globin heated at 80°C was 2.3×10^7 and that of the peak fractions was 2.4×10^6 . The MW of globin heated at 90°C was extremely high (4×10^8).

Figure 6 shows the ellipticity at 222 nm, the relative ratio of aggregates measured by three different methods and the intrinsic viscosity of globin heated at various temperatures. All the relative ratios of aggregates rapidly increased around 50°C, and the tendency to increase coincided with the decrease of the negative

FIG. 7. Electron Micrographs of Hemoglobin and Globin Heated at Various Temperatures.

a) globin heated at 60°C, b) globin heated at 80°C, c) globin heated at 90°C, d) hemoglobin heated at 80°C. All bars indicate 200 nm.

ellipticity. However, the rapid increase in the intrinsic viscosity which occurred above 80°C was not coincident with an increase in the relative ratio of aggregates.

The physicochemical results for heated globin were supplemented by electron microscopic observation. Figure 7 shows electron micrographs of heated globin and hemoglobin. Soluble aggregates of globin formed by heating at 60°C were nearly globular particles with a diameter of 15 to 20 nm (Fig. 7a). On the other hand, those formed by heating at 80°C were like strands with a thickness of 8 to 10 nm (Fig. 7b). The globin heated at 90°C formed highly entangled fibrous aggregates with a thickness of 8 to 10 nm (Fig. 7c). The high concentration dependence of reduced viscosity in Fig. 4 was considered to be attributable to the entanglement of the fibrous globin aggregates. The latter were also observed when the globular particles formed by heating at 60°C were further heated at 90°C (not shown but almost the same as in Fig. 7c). Electron micrographs indicated that the linear aggregation predominantly occurred at the higher heating temperature (above 80°C). Hemoglobin heated at 80°C formed aggregates consisting of globular aggregates with a diameter of 15 to 25 nm, and larger aggregates composed of a number of globules (Fig. 7d).

DISCUSSION

The mean residue ellipticity of bovine globin prepared by the CMC procedure [globin (CMC)] was $-10,840$ at 222 nm (Fig. 1). Vodrazka *et al.*²⁰⁾ showed that the ellipticity of bovine hemoglobin and native globin prepared by an acidified acetone procedure [globin (acetone)] at 222 nm was $-24,500$ and $-17,500$, respectively, which corresponded to 73% and 49% α -helix contents calculated with the formulas of Chen and Yang.²¹⁾ They speculated that the lower helix content of globin compared to hemoglobin is due to the unfolding of regions that in hemoglobin are stabilized by interaction with the heme group. The α -helix content of globin (CMC) was 28% when calcu-

lated with the formulas of Chen and Yang.²¹⁾ This helix content was considerably lower than that of native bovine globin (acetone). It was shown that the native bovine globin obtained from the acidified acetone globin fraction by removal of the denatured molecules which were insoluble in a neutral medium represented approx. 10% of the globin fraction.²⁰⁾ It was shown that a decrease in pH resulted in the unfolding of the human globin molecules,²²⁾ and a major loss of about 35% helicity of water buffalo apomyoglobin occurred between pH 5.6 and 4.3.²³⁾ Therefore, the lower helicity of globin (CMC) was due to the presence of denatured molecules and to the ellipticity at the slightly acid pH. Although sedimentation coefficients of globin (CMC) were somewhat large (3.7 *S* at pH 5.3, 2.7 *S* at pH 4.8) (Table I) compared to globin (acetone) (3.1 *S* at pH 6.2, 2.7 *S* at pH 4.8),²⁰⁾ globin (CMC) contained no aggregates (Figs. 2, 3). It has been considered that the acidified acetone procedure is a mild method for preparing globin, and hemoglobin with properties quite similar to those of the original hemoglobin can be reconstituted from globin (acetone) and heme.²⁴⁾ Therefore, the CMC procedure was also considered to be a mild method to prepare globin.

A great decrease in negative ellipticity of heated globin was observed between 45 and 50°C (Fig. 1). It was suggested that unfolding of globin molecules may occur at this temperature. The temperature of half-precipitation caused by thermal denaturation of native human globin was reported to be around 40°C.^{25,26)} The high denaturation temperature of globin in the present study may be attributable to the short heating time (10 min) and low pH (5.3), since the heating conditions for native human globin were 60 min at pH 7. Soluble aggregates were also formed between 45 and 50°C (Fig. 6). Therefore, it is considered that the formation of soluble aggregates may occur spontaneously with the unfolding of globin molecules.

The soluble aggregates were globular particles with diameters ranging from 15 to 20 nm

(Fig. 7a) and their sedimentation coefficient and molecular weight were approx. $20S$ and 8×10^5 , respectively (Table I, Fig. 5). It was estimated that a globular aggregate consisted of about fifty globin molecules with a molecular weight of about 16,000.²⁷⁾ It was found that the globin was less stable than the hemoglobin, and the presence of aromatic and alicyclic hydrocarbons increased the resistance of the globin to heat denaturation.²³⁾ The role of the hydrophobic bond in the slow and progressive aggregation of globin molecules at pHs above 3 has also been considered by Rossi-Franelli *et al.*²⁴⁾ Therefore, it is considered that aggregation of globin molecules is due to their interaction with the hydrophobic regions exposed by the separation of the heme. It is also suggested that the interchain disulfide bond plays a minor role in forming an aggregate because bovine globin possesses no intramolecular disulfide bond and only β -globin has one sulfhydryl group²⁷⁾ resulting in formation of dimeric β -globin. About two thirds of the globin molecules formed soluble aggregates within 30 sec on heating at 60 or 70°C and their sedimentation coefficient showed only a slight increase after prolonged heating up to 30 min (data not shown). It may be considered that the definite size of the globular aggregates is due to their definite interactive surface area such as the hydrophobic regions of unfolded molecules and to little interaction between aggregates, because the hydrophobic regions on the surface of unfolded molecules are buried inside during the formation of aggregates.

The globin heated at 90°C formed fibrous macro-aggregates with a thickness of 8 to 10 nm (Fig. 7c), and with an extremely high molecular weight (Fig. 5) and a large sedimentation coefficient (Table I). Their solution was also extremely viscous. These huge aggregates in solution seem to be held by the critical balance between attractive and repulsive forces. Clark *et al.*²⁸⁾ showed that linear aggregates were predominantly observed on electron microscopy in the aggregates of bovine serum albumin formed at sub-gelling con-

centrations of protein and under the same conditions as produced the homogeneous gel networks. Tombs²⁹⁾ suggested that strands must be formed by the aggregates to form a gel, and random aggregation would presumably lead to more or less spherical particles. It appears likely that hemoglobin aggregates randomly and globin forms strands (Fig. 7c, d). Thoms³⁰⁾ also suggested that globular proteins form gels as a result of aggregation to form strands followed by interaction of the strands to form the gel mesh. Hermansson and Buchheim³¹⁾ found that the network structure of soy protein gel was formed at two levels, one of them being orientation on the molecular level in the form of strands. The globin can form a transparent gel at a higher concentration under the same heat conditions as in the present study,¹³⁾ so it is assumed that the three dimensional network formation of globin gel is formed from highly entangled fibrous macro-aggregates.

The thermal induced gelation of globin speculated from the present results is as follows. At first, globular aggregates with a molecular weight of approx. 8×10^5 are formed spontaneously with the unfolding of globin molecules. Secondly, the globular aggregates extend and associate to form fibrous macro-aggregates with a high molecular weight of about 10^8 . Finally, the fibrous macro-aggregates form a three dimensional network leading to formation of a gel. Mori *et al.*³²⁾ also found that heat produced soluble aggregates as transient intermediates with a molecular weight 8×10^6 for soy protein. Therefore, it appears likely that globular protein tends to form heat-induced soluble aggregates as an intermediate form under appropriate conditions.

However, it is not clear that the formation of intermediates like globular aggregates is indispensable for formation of fibrous macro-aggregates. It is not clear that globular and fibrous aggregates of the same size as formed at a sub-gelling concentration can be formed at the higher concentrations that produced the gel network. Therefore, further studies must be

carried out on a very early stage of aggregation under the conditions of a higher protein concentration and high temperature.

Acknowledgments. The support of this project by the Ryoshoku Kenkyukai, Japan, is gratefully acknowledged. We also thank the Center for Chemical and Physical Analysis of the National Institute for Basic Biology for the use of the light scattering photometer.

REFERENCES

- 1) P. T. Tybor, C. W. Dill and W. A. Landman, *J. Food Sci.*, **38**, 4 (1973).
- 2) P. T. Tybor, C. W. Dill and W. A. Landman, *J. Food Sci.*, **40**, 155 (1975).
- 3) J. Wismer-Pedersen, *Fleischwirtsch.*, **60**, 987 (1980).
- 4) G. Drepper and K. Drepper, *Fleischwirtsch.*, **59**, 252 (1979).
- 5) Y. Sato, S. Hayakawa and M. Hayakawa, *J. Food Technol.*, **16**, 81 (1981).
- 6) M. D. Ranken, "Applied Protein Chemistry," ed. by R. A. Grant, Applied Science Publ., Ltd., 1980, p. 169.
- 7) A. V. Kuppevelt, G. Levin and P. Reizenstein, *Nutri. Rep. Int.*, **13**, 429 (1976).
- 8) W. A. Landman, C. W. Dill and C. R. Young, *J. Nutr.*, **110**, 2254 (1980).
- 9) E. L. Parmer, J. G. Surak and F. W. Knapp, *J. Food Sci.*, **43**, 499 (1978).
- 10) C. Devuono, M. Penteado, F. M. Lajolo and N. Pereira dos Santos, *J. Sci. Food Agric.*, **30**, 809 (1979).
- 11) J. E. Kinsella, *CRC Crit. Rev. Food Sci. Nutr.*, **4**, 219 (1976).
- 12) D. D. Crenwelge, C. W. Dill, P. T. Tybor and W. A. Landman, *J. Food Sci.*, **39**, 175 (1974).
- 13) S. Hayakawa, T. Ogawa and Y. Sato, *J. Food Sci.*, **47**, 1415 (1982).
- 14) H. A. Caldironi and H. W. Ockerman, *J. Food Sci.*, **47**, 405 (1982).
- 15) F. W. Knapp, R. H. Schmidt, W. J. Mauldin and E. M. Ahmed, *J. Food Protection*, **41**, 257 (1978).
- 16) T. H. J. Huisman and A. M. Dozy, *J. Chromatogr.*, **7**, 180 (1962).
- 17) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 18) B. Bull and K. Breese, *Arch. Biochem. Biophys.*, **158**, 681 (1973).
- 19) E. Stellwagen and H. Wilgus, *Nature*, **275**, 342 (1978).
- 20) Z. Vodrazka, Z. Hrkál, M. B. Kodicek and D. Jandora, *Eur. J. Biochem.*, **31**, 296 (1972).
- 21) Y. H. Chen and J. T. Yang, *Biochem. Biophys. Res. Commun.*, **44**, 1285 (1971).
- 22) Z. Hrkál and Z. Vodrazka, *Biochim. Biophys. Acta*, **133**, 527 (1967).
- 23) G. Colonna, G. Irace, G. Parlato, S. M. Aloj and C. Calesterieri, *Biochim. Biophys. Acta*, **532**, 354 (1978).
- 24) A. Rossi-Fanelli, E. Antonini and A. Caputo, *Advance in Protein Chemistry*, **19**, 73 (1964).
- 25) J. Sojika, H. Hrkál and Z. Vodrazka, *Collect. Czech. Chem. Commun.*, **39**, 509 (1974).
- 26) M. Kodicek and Z. Vodrazka, *Collect. Czech. Chem. Commun.*, **41**, 1811 (1976).
- 27) M. O. Dayhoff, "Atlas of Protein Sequence and Structure," Vol. 5, National Biomedical Foundation, Washington D. C., 1972.
- 28) A. H. Clark, F. J. Judge, J. B. Richards, J. M. Stubbs and A. Suggett, *Int. J. Peptide Protein Res.*, **17**, 380 (1981).
- 29) M. P. Tombs, "Protein as Human Food," ed. by R. A. Lawrie, Butterworth Publ., 1970, p. 126.
- 30) M. P. Tombs, *Faraday Discuss. Chem. Soc.*, **57**, 158 (1974).
- 31) A. M. Hermansson and W. Buchheim, *J. Colloid Int. Sci.*, **81**, 519 (1981).
- 32) T. Mori, T. Nakamura and S. Utsumi, *J. Food Sci.*, **47**, 26 (1981).