Reactivity of Sulfhydryl and Disulfide in Proteins. VI. Effect of Heat Denaturation of Bovine Serum Albumin (BSA) on Sulfhydryl and Reactive Disulfide Content[1]

I. M. Kolthoff and B. H. Tan

Contribution from the School of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received February 5, 1965*

Upon heating in the absence of oxygen of 1% albumin solutions at 70° or higher temperatures in buffers with pH between 5 and 8, intermolecular exchange (in addition to intramolecular exchange) occurs between sulfhydryl and disulfide groups, resulting in the formation of polymer molecules. The apparent sulfhydryl content decreases continuously on heating. However, all sulfhydryl groups present in the unheated protein are found to react when the titration is carried out in 8 M urea. The number of reactive disulfide groups increases from practically 0 to 9 after heating for 1 hr. at 70°. When heated in the presence of a sulfhydryl binding agent in a ratio of 1 equiv. of reagent to 1 equiv. of sulfhydryl, intramolecular hydrogen bonds are broken and intermolecular hydrogen bonds are formed, resulting in agglomeration of protein molecules. Reactive disulfide increases from 0.2 to 5 upon heating in the presence of a mercurial at pH 6.8 to 8.5. When such a heated solution is made 8 M in urea and then diluted ten times, the number of reactive disulfide groups becomes practically zero and equal to that in unheated albumin. In this sense the heat aggregation in the presence of a mercurial is reversible.

From viscosity and sedimentation characteristics which accompany the thermal denaturation at 100° of dilute solutions of bovine serum albumin (BSA) Hospelhorn and Jensen[2] concluded that sulfhydryl in BSA promotes lateral association of protein molecules through a chain reaction with disulfide groups during heat denaturation. On the other hand, when the sulfhydryl in BSA is removed or blocked by silver, a different kind of aggregation on heat denaturation occurs. The authors suggested that a relatively slow reaction of unknown nature occurs, which causes the linking of protein chains at relatively few points in the molecule to form a regular three-dimensional gel network. In order to gain further insight into the mechanism of the two aggregation reactions, we have determined the change of sulfhydryl content of BSA during heat denaturation and the amount of "reactive disulfide" formed when the sulfhydryl group was free or blocked. Reactive disulfide is defined by the number of disulfide groups which react with sulfite under conditions specified in the procedures.

Marked changes of apparent sulfhydryl and reactive disulfide contents were found upon heat denaturation in the pH range between 5 and 8.5. In order to substantiate the interpretation of the results, the intrinsic viscosity of the heated protein was measured under various conditions. It is concluded that the agglomerated protein, formed when the sulfhydryl group is blocked, can be completely deagglomerated in 4 *M* guanidine hydrochloride or 8 *M* urea with reformation of protein with the same sulfhydryl and reactive disulfide content as the native albumin. Such a reversal cannot occur when the sulfhydryl is not blocked during the heating, as the protein molecules become chemically linked together.

Experimental

Bovine Serum Albumin (BSA). Most of the experiments were carried out with Lot A 69805 of albumin, but several experiments have also been done with Lot V 68802, both lots having been obtained from the Armour Laboratories. The former contained 0.80 mole sulfhydryl and the latter 0.67 mole sulfhydryl in the native state.

Determination of Sulfhydryl. Usually, this was determined by amperometric titration with silver nitrate in Tris (pH 7.4–8.1) or ammonia buffer (pH 9.2) using the rotated platinum electrode as indicator electrode at −0.3 to −0.6 v. *vs.* s.c.e. In several instances amperometric titrations were also carried out in the same buffers at −0.6 v. with ethylmercuric chloride using a rotated dropping mercury electrode as indicator electrode. The results obtained by the various methods agreed within a few per cent.

Reactive Disulfide. Procedure a. After heating, the protein was diluted to 0.1 to 0.2% in 0.2 *M* ammonia buffer of pH 9–9.2, made 0.05 to 0.1 *M* in sodium sulfite, and titrated amperometrically at −0.4 to −0.6 v. *vs.* s.c.e. with silver nitrate, using the rotated platinum wire electrode as indicator electrode. In several instances the results were checked by titration with ethylmercuric chloride at −0.8 to −0.9 v. using a rotated dropping mercury electrode (r.d.m.e.) as indicator electrode.

Procedure b. To 20 ml. of air-free 1–1.25% protein solution was added 2.5 ml. of 1 *M* sodium sulfite solution to make its concentration 0.1 *M* and 2.5 ml. of 1 *M* perchloric acid to a pH of 6.3, using the glass electrode as indicator electrode. After standing for about 1 hr., 5 ml. of the mixture was added to 45 ml. of air-free 0.022 *M* perchloric acid to give a pH of 2, and the sulfhydryl was then titrated amperometrically in a nitrogen atmosphere with mercuric chloride or ethylmercuric chloride at the r.d.m.e. at −0.3 v. *vs.* s.c.e. Under these conditions the sulfhydryl in native BSA reacts with mercuric chloride in a molecular ratio of 1:1. The liberated sulfhydryl from disulfide groups

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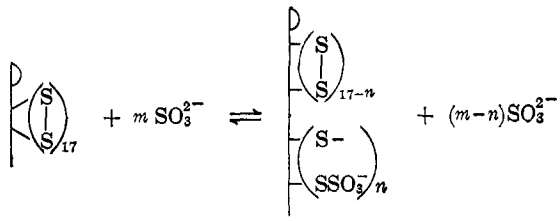
(2) V. D. Hospelhorn and E. V. Jensen, *J. Am. Chem. Soc.*, **76**, 2830 (1954); see also E. V. Jensen, V. D. Hospelhorn, D. F. Tapley, and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950).

reacts in a ratio of 2:1. The results were checked by amperometric titration with ethylmercuric chloride at a rotated dropping mercury electrode at −0.35 v. *vs.* s.c.e. "Reactive disulfide," as defined by the above conditions, is very small in native albumin. In a subsequent paper it will be reported that the sum of moles of sulfhydryl and "reactive disulfide" in various lots of bovine serum albumin has been found to be equal to 1 by both procedures a and b. In the present work "reactive disulfide" was found to increase considerably upon heat denaturation. The results by procedure b were usually some 10 to 15% smaller than by procedure a. The reason is that in procedure b the equilibrium number of disulfide groups is determined which reacts at the (optimum) pH of 6 with sulfite.



In procedure a the equilibrium value at pH 9 is smaller that at pH 6. However, during the titration the equilibrium is displaced to the right, but the amount of "reactive disulfide" thus found is close to that found by procedure b. Procedure a was generally used as it is much simpler than procedure b.

Total Disulfide. This was determined as described previously in 8 *M* urea or 4 *M* guanidine hydrochloride.[3]

Heat Denaturation. Unless stated otherwise, oxygen-free approximately 1% albumin solutions were heated at 70° for a given time and cooled to room temperature. The viscosity was then measured at 25°. The solutions were diluted under oxygen-free conditions to determine sulfhydryl and reactive disulfide. In several instances the reduced viscosity of the protein after heating was determined by diluting with an equal volume of 8 *M* guanidine hydrochloride (GHCl) or by making it 8 *M* in urea by adding the proper amount in the solid form.

Viscosity. The same procedure was used as described previously.[4] Results are expressed in terms of reduced viscosity.

Results

Sulfhydryl. (*a*) *Heat Denaturation in the Absence of Oxygen.* From Table I it is clear that the apparent sulfhydryl content decreases with time of heating at 70° at pH 7. In order to establish whether sulfhydryl actually disappeared or whether it was masked in the heated protein (A 69805), it was determined under air-free conditions by making the solution after heating 7–8 *M* in urea and titrating with silver nitrate or ethylmercuric chloride in Tris buffer of pH 8.0. Under these conditions the amount of sulfhydryl present in the unheated BSA was found back. Upon heating at pH greater than 8.5 apparent sulfhydryl increases as a result of hydrolysis of disulfide groups.

(3) I. M. Kolthoff, A. Anastasi, and B. H. Tan, *J. Am. Chem. Soc.*, **80**, 3235 (1958).
(4) I. M. Kolthoff, A. Anastasi, W. Stricks, B. H. Tan, and G. S. Deshmukh, *ibid.*, **79**, 5102 (1957).

Table I. Sulfhydryl after heating 1% Albumin Solution in 0.04 *M* Phosphate Buffer at pH 6.8–7.0, at 70° in Nitrogen

Heating, hr.	—SH per mole of protein— Lot V 68802	Lot A 69805
0	0.67	0.80
0.25	0.57	
1	0.54	0.73
3	0.44	0.59
5.5	0.32	
4[a]		0.86
3[b]		1.2

[a] pH 8.5. [b] pH 9.3.

(*b*) *Heat Denaturation in the Presence of Air or Ferricyanide.* Sulfhydryl decreased much more rapidly upon heating at 70° in the presence of oxygen or 3 moles of ferricyanide/mole of sulfhydryl. Although sulfhydryl is not oxidized when a 1% albumin solution is allowed to stand at 25° in the presence of oxygen (air-saturated) or of 3 moles of ferricyanide/mole of sulfhydryl, it decreases very rapidly under the same conditions at 70°. For example, sulfhydryl in the presence of oxygen decreased at pH 7.0 from 0.67 (Lot V 68802) to 0.20 after 1 hr. at 70°, and to 0.07 after 2 hr. In the presence of ferricyanide it decreased to 0.33 after 15 min., to 0.16 after 30 min., to 0.07 after 1 hr., and to 0 after 2 hr. Experiments with Lot A 69805 qualitatively yielded similar results. For example, after 3 hr. of heating at 70° at pH 6.8, sulfhydryl corresponded to 0.07 mole of SH/mole of albumin. When titrated in 8 *M* urea with ethylmercuric chloride, the sulfhydryl content was 0.18 mole. Evidently, under the above conditions sulfhydryl is oxidized to disulfide upon heating in the presence of oxygen or ferricyanide.

Reactive Disulfide. Reactive disulfide was determined after various periods of heating in the absence of oxygen in phosphate buffers of pH 6.9, 7.6, and 8.5, respectively. Similar experiments were carried out in the presence of 1 equiv. of mercurial or silver nitrate

Table II. Reactive Disulfide after Heating 1–1.2% Albumin Solution at 70° in Phosphate Buffers[a]

Heating, hr.	pH	Addition before heating	Expt. no.	—Reactive disulfide, moles/mole of albumin— Procedure a	Procedure b
0.5	6.9	....	2	6.4 ± 0.2	
1	6.9	....	5	8.8 ± 0.5	7.5 ± 0.3
2	6.9	....	5	9.2 ± 0.5	
3	6.9	....	2	7.8 ± 0.4	
1	6.9	$1HgCl_2$[b]	2	4.3 ± 0.2	4.0 ± 0.5
1	6.9	$1C_2H_5HgCl$[b]	2	4.5 ± 0.3	4.2 ± 0.5
2	6.9	$1C_2H_5HgCl$[b]	2	5.0 ± 0.5	
1	6.9	$1AgNO_3$[b]	3	4.6 ± 0.4	
2	6.9	Air-saturated	3	6.8 ± 0.5	
1	7.6	....	2	5.9 ± 0.5	
2,4	7.6	....	2	6.1 ± 0.5	
1	7.6	$1AgNO_3$[b]	2	4.4 ± 0.2	
2,4	7.6	$1AgNO_3$[b]	2	5 ± 0.2	
1	8.5	....	2	5.2 ± 0.2	
3	8.5	....	2	6.4 ± 0.2	
1	8.5	$1AgNO_3$[b]	1	5.0	
3	8.5	$1AgNO_3$[b]	1	5.3	

[a] Lot A 69805; before heating SH = 0.80 mole; reactive disulfide = 0.2 mole. [b] Moles added per mole of sulfhydryl in BSA.

Table III. Heat Denaturation of BSA (Lot V 68802) in the Presence and Absence of 1 Equiv. of Mercurial

Heating, hr.	pH	No $HgCl_2$ Appearance	No $HgCl_2$ η_{red}[a]	No $HgCl_2$ η in 4 *M* GHCl[b]	1 $HgCl_2$ Appearance	1 $HgCl_2$ η_{red}[a]	1 $HgCl_2$ η in 4 *M* GHCl[b]
2.5	6.0	Turbid, ppt.	0.5	0.67	Precipitate		
0.3					Turbid, ppt.	(>1)	0.18
1.5					Precipitate	(>1)	0.18
1	6.6	Turbid	0.15	0.26			
3		Turbid	0.27	0.42			
0.3					Turbid	0.4	0.18
2					Turbid	>1	0.18
1	7.0	Weak bluish (opalescence)	0.1	0.18	Bluish	0.30	0.17
2		Strong bluish	0.12	0.20	Bluish	0.40	0.17
4		Strong bluish	0.15	0.22			
1	7.9	Weak bluish	0.060		Bluish	0.048	0.175
4		Weak bluish	0.10		Bluish	0.050	0.175
19		Weak bluish	0.18	0.31	Bluish	0.058	0.175

[a] η of unheated BSA 0.045. [b] η of unheated BSA 0.175.

referred to the sulfhydryl content. Only a few experiments were carried out at pH 7.6 in the presence of oxygen and absence of sulfhydryl binding agent. The results are given in Table II. In the absence of a sulfhydryl binding agent there is a rapid increase of reactive disulfide from 0.2 in the native albumin to 9 after 1 hr. of heating at pH 6.9. On further heating the reactive disulfide hardly changes, and it starts to decrease slowly after 3 hr. In experiments carried out at 80, 90, and 100° the same pattern was observed, the reactive disulfide increased more rapidly, and the maximum was attained more rapidly with increasing temperature. In the presence of a sulfhydryl binding agent the formation of reactive disulfide is slower than in its absence and the maximum amount is smaller. The difference in rate of formation in the presence and absence of sulfhydryl bonder is clearly illustrated in Figure 1, where the rates of formation of reactive disulfide at 65 and 70° are compared in a phosphate buffer of pH 6.8 in the presence and absence of 1 equiv. of ethylmercuric chloride.

Several experiments were carried out in which a 1.5% albumin solution after heating at 70° in a phosphate buffer of pH 6.8 in the presence of 1 equiv. of silver nitrate was made 7 *M* in urea by adding the calculated amount of solid urea. Five milliliters of the solution thus obtained was then diluted with 45 ml. of ammonia buffer to reverse the urea denaturation and reactive disulfide was determined by procedure a. Blank experiments were carried out with native (unheated) albumin. Although in 7–8 *M* urea all 16 ± 1 disulfide groups in unheated albumin are reactive toward sulfite according to procedure a, the amount of reactive disulfide found after ten times dilution was the same as in the original sample, 0.2 mole in the lot used by us. The dilution was made after standing in 7 *M* urea for 10 to 15 min. Upon longer standing cross linking occurs and reactive disulfide increases. For example, after 1 hr. standing before dilution, reactive disulfide was 0.7 mole instead of 0.2 mole. When heated for 2.5 hr. in the absence of a sulfhydryl binding agent and then treated with urea followed by dilution according to the above procedure, 8.0–9.0 reactive disulfide groups were found, about the same as without urea treatment after the heating. When 1 equiv. of silver nitrate was present during the 2.5 hr. of heating and the solution was treated as above, reactive disulfide was only 0.7–0.8 as compared to 5.0 without urea treatment.

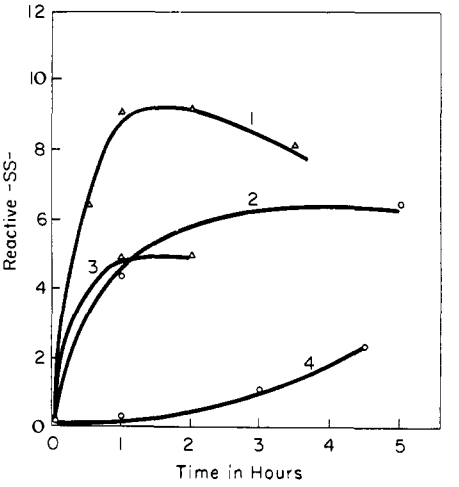


Figure 1. Reactive disulfide upon heating 1% BSA in phosphate buffer of pH 6.8 at 70 and 65°: 1, at 70°, no addition of mercurial; 2, as 1 at 65°; 3, at 70° with 1 equiv. of C_2H_5HgCl; 4, as 3 at 65°.

Intrinsic Viscosity. Rapid precipitation occurred upon heating albumin solution in a 0.02 *M* acetate buffer at pH 4.9 either in the absence or in the presence of 1 equiv. (to SH) of mercuric chloride or ethylmercuric chloride. After heating and cooling, the mixtures were diluted twice with 8 *M* guanidine hydrochloride (GHCl) solutions. Mixtures obtained in the absence of a mercurial remained turbid in the GHCl solution. However, clear solutions were obtained when 1 equiv. of mercurial had been present during the heat denaturation. The intrinsic viscosity of such a solution obtained after 1 hr. of heating at 70° and pH 4.9 was 0.22 as compared to 0.175 for unheated BSA in 4 *M* GHCl. All mixtures, obtained after heating either in the absence or presence of mercurial, became clear in 4 *M* GHCl which was 0.03 *M* in sulfite. The viscosity of such solutions was 0.42, the same as that of unheated BSA in the same medium. Evidently, there was complete depolymerization of heated protein in this medium.

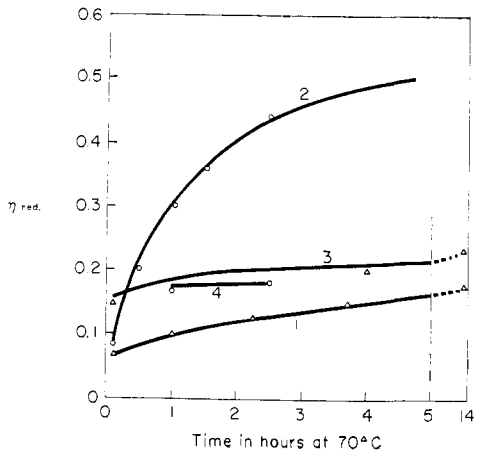


Figure 2. Reduced viscosity after heating at 70° of 1% albumin in 0.04 *M* phosphate buffer at pH 7: 1, no addition; 2, with 1 mole of $HgCl_2$/mole of SH; 3, as 1, in 4 *M* GHCl, after heating; 4, as 2, in 4 *M* GHCl, after heating.

A brief summary of results obtained upon heating in phosphate buffers in the pH range between 6 and 8 is given in Table III. As an illustration the difference in behavior at pH 7 in the presence and absence of mercurial is presented in Figure 2. Many experiments were carried out in the pH range from 6 to 7 in which 1% albumin solutions were heated at 70° for 15 min. or longer. Upon addition of 1 equiv. of mercurial after 15 min. or longer heating and continued heating, the mixtures behaved quite differently from those with mercurial present at the start. For example, after 15 min. at pH 7 and 70° followed by addition of 1 equiv. of mercuric chloride, the intrinsic viscosity did not increase sharply on further heating, but increased very slowly from 0.05 to 0.08 after 3 hr. However, the mercurial prevented further cross linking, as was evident from the viscosity in 4 *M* GHCl. This was 0.17 after 15 min. of heating and 0.18 after 3 hr. continued heating in the presence of 1 equiv. of mercurial. Also, the viscosity after 3 hr. of heating in the absence of mercurial was 0.135, as compared to 0.08 when the mercuric chloride was added 15 min. after starting the heating.

Discussion

When heated in the absence of a sulfhydryl binding agent, cross linking occurs by intermolecular exchange between sulfhydryl and disulfide groups. Thus polymers are formed which precipitate at or near the isoelectric pH, precipitation decreasing with increasing pH. No precipitation occurs at pH equal to or greater than 8. The rate of the sulfhydryl–disulfide exchange increases with increasing pH, but the rate of the intermolecular exchange decreases, because of increasing negative charge and repulsion between the protein molecules. When the heated polymerized protein solutions are made 4 *M* in guanidine hydrochloride (GHCl), the intrinsic viscosity is found greater than that of unheated albumin in the same medium. The intermolecular cross linking is accompanied by intramolecular exchange between sulfhydryl and disulfide. As a result of the cross linking and the intramolecular exchange, not all the sulfhydryl groups are available for reaction on direct titration with silver nitrate or a mercurial (Table I). However, after partial uncoiling of the heated protein in 8 *M* urea, amperometric titration yields the original sulfhydryl content.

The large increase of the number of reactive disulfide groups on heating (Table II) is partly due to a structural change with stretching of the polymer molecules which accompanies the intermolecular and the intramolecular exchange, and partly due to reshuffling of hydrogen bonds (*vide infra*). When heated in the presence of a sulfhydryl binding agent in a molar ratio of one reagent to one sulfhydryl, the exchange between sulfhydryl and disulfide is prevented. Under these conditions intramolecular hydrogen bonds are broken and intermolecular hydrogen bonds are formed, resulting in agglomeration and increase in viscosity. This agglomeration is promoted by attraction between positive groups and negative groups in the different protein molecules, the electrostatic attraction decreasing with increasing negative charge. As a result there is practically no agglomeration at pH 8 (Table III). The agglomeration at pH 5–8 is physical in nature, as is evident from the fact that the viscosity, upon making the mixture after heating 4 *M* in GHCl, is found to be the same as that of unheated albumin in the same medium (Table III). When heated in the presence of 1 equiv. of mercurial or silver nitrate, the number of reactive disulfide groups increases from 0.2 (in our sample) to 5, the same value being found at pH 6.8 as at pH 8.5, even though at the higher pH no intermolecular hydrogen bonding accompanied by agglomeration occurs. Thus the increase in reactive disulfide at pH 5 to 8.5 must be attributed to intramolecular reshuffling of hydrogen bonds accompanied by loosening of the structure. It is of special interest to note that the reactive disulfide becomes practically equal again to that in the native albumin when the heated protein solution is made 8 *M* in urea and then diluted ten times. Apparently the original structure (intramolecular hydrogen bonding) of the albumin is then restored, and the heat denaturation reaction in the presence of a sulfhydryl binding agent may be considered as reversible under the above conditons. However, when heated in the absence of a sulfhydryl binding agent, the heat denaturation is irreversible and the molecules remain polymerized in 8 *M* urea or 4 *M* GHCl. Also when after treatment with 8 *M* urea the solution is diluted ten times, the number of reactive disulfide groups after heating for 2 hr. at 70° at pH 7 remains equal to 9, and thus is not reduced by this treatment. Complete depolymerization can be brought about in 8 *M* urea or 4 *M* GHCl when sulfite in a concentration of 0.03 to 0.05 *M* is added, which results in a breaking of the inter- and intramolecular disulfide bonds. The effect of preheating for 15 min. at 70° and pH 7 before adding mercurial is striking. Upon further heating there is no longer a large increase in viscosity, as observed when mercurial is added at the start of heating (see curve 2 in Figure 2), and no agglomeration occurs. However, upon continued heating, further exchange between sulfhydryl and disulfide does not occur, as is evident from the normal value of the viscosity in 4 *M* GHCl after heating for 2–3 hr. The exchange reaction must be very rapid, because after 15 min. of heating at pH 7 in the absence of mercurial the number of disulfide groups has increased from 0.2 to 5.