[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

A Study of the Reaction of the Disulfide Groups of Bovine Serum Albumin during Heat Denaturation[1]

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The polymerization of heat denatured bovine serum albumin has been followed by means of solubility in 83% acetic acid. The monomer and lower polymers are soluble in this solvent while the higher polymers are not. The effects of *p*H changes and sulfhydryl reagents indicate that the polymerization proceeds by means of a disulfide exchange with a sulfhydryl intermediate. A sample of a mixture of monomer and dimer was obtained in water-soluble form by the lyophilization of an 83% acetic acid solution. This recovered protein had slightly higher intrinsic viscosity than the native protein, slightly lower optical rotation and a more rapid rate of digestion by trypsin.

Previous work on the recovery of bovine serum albumin (BSA) from heat denaturation,[4,5] from acid denaturation[6,7] and from urea denaturation[8] has indicated that the amount of recoverable or non-recoverable material depends upon the nature and extent of the denaturing conditions. The denatured precipitated protein usually was treated with strong acid or alkali, and the part that would dissolve was called the recoverable portion.

Heat denaturation has been chosen for this work because it offers the easiest, quickest and most complete removal of the denaturing conditions. The previous paper[9] has shown that BSA is not degraded in acetic acid solution. This provides a convenient method for redissolving heat coagulated BSA. The acetic acid may be removed easily by lyophilization, and the recovered protein may be studied.

Experimental

Materials.—The BSA used was part of Pentex Lot A1202, the preparation and properties of which are described in the previous paper.[9] All other materials used were reagent grade chemicals.

Methods. Denaturation.—The protein was denatured by plunging a thin walled test-tube containing 1 ml. of a 0.6% aqueous solution of BSA into a bath of boiling water. Denaturation was halted by removing the test-tube from the boiling water-bath and quickly blowing 5 ml. of cold glacial acetic acid from a pipet into the test-tube. The insoluble portion of the coagulated protein was removed by centrifugation. The contents were centrifuged at 2,000 times gravity for 2 hr. to pack the gelatinous coagulum. The contents were then stirred to remove the concentration gradients in the supernatant and were centrifuged again at 100 times gravity to settle the solid material. The concentration of the soluble protein was read in the Beckman DU spectrophotometer from the absorption at 278 mμ assuming an optical density of 6.63 for a 1% protein solution.

Recovery of Denatured BSA.—The protein was denatured in the manner just described. The material which was soluble in 5 volumes of acetic acid was recovered by freezing and lyophilizing the acetic acid solution. Protein thus recovered from heat denaturation (recovered-BSA) was completely soluble in distilled water if denaturation had not proceeded longer than about 30 sec.

(1) Taken from part of the Ph.D. thesis of L. K. Steinrauf, University of Washington, 1957.

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(3) The authors are grateful for financial support from U. S. Public Health Service Grant #H2217.

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Results

Precipitation of BSA by Heat.—BSA will precipitate from aqueous solution near the isoelectric point (4.8) almost immediately at 100°. This precipitate was completely soluble in 5 volumes of acetic acid if the acid was added within 30 sec. from the start of heating. After 30 sec. the amount of acetic acid soluble protein decreased rapidly depending on the *p*H. In the *p*H range studied (4.7 to 7.2) the rate of formation of insoluble material was found to be most rapid at *p*H 5.6. Results showing the decrease in the amount of soluble protein with time as measured by optical density are given in Fig. 1 for 0.6% BSA in distilled water and in 0.1 *M* sodium chloride at *p*H 5.6.

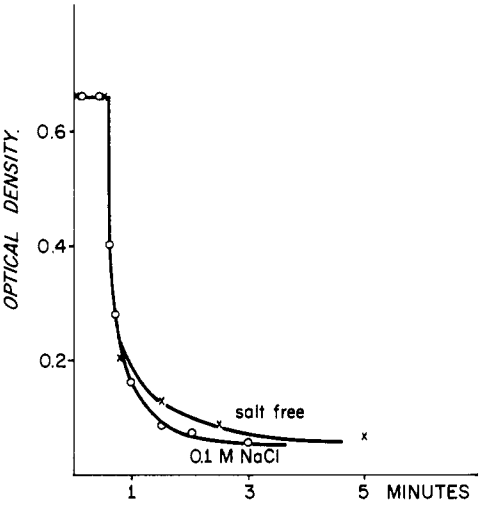

Fig. 1.—Decrease in the amount of acetic acid soluble BSA coagulum with time of heating as measured by the optical density of the supernatant at 278 mμ.

When 9.3×10^{-5} *M* mercuric acetate or mercuric chloride was added to the protein solutions (4.6×10^{-5} *M* in protein) the precipitate remained soluble in 5 volumes of acetic acid even after prolonged heating. When 2.3×10^{-4} *M* cysteine was added, the protein solutions immediately formed a gel at 100° which could not be broken up by centrifugation. Mercuric ions present at 2.3×10^{-4} *M* prevented the protein from being precipitated from aqueous solutions at 100° by prolonged heating. Salts of silver, zinc or calcium had no such protective effects.

Recovery of Heat Coagulated BSA.—Figure 2 shows the sedimentation patterns of BSA in aque-

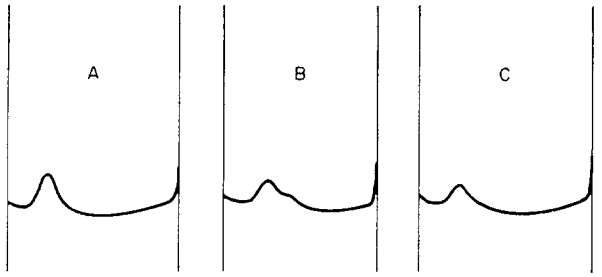

Fig. 2.—Sedimentation patterns of BSA in aqueous acetate buffer. The protein was heated for various times and then recovered by the addition of acetic acid and lyophilization. Time of heating: (A) 0 sec.; (B) 15 sec.; (C) 33 sec. Concentration of soluble protein was about 0.6% in all cases. The pictures were taken 56 min. after reaching 59,780 r.p.m. with a bar angle of 60°.

ous solutions after it had been coagulated by heating for various lengths of time and then redissolved by the addition of 5 volumes of acetic acid. The soluble material was recovered by lyophilization and dissolved in an aqueous solution of 0.05 *M* sodium acetate, 0.05 *M* acetic acid, 1.0 *M* sodium chloride, *p*H 4.7. Figure 2A shows the sedimentation pattern of the native protein. Figure 2B is for a protein solution that was coagulated by heating for 15 sec. The time of heating was not sufficient to cause the protein to become insoluble in acetic acid. Figure 2C is for a protein solution that was heated for 33 sec. During this time denaturation had advanced to a degree where much of the coagulum remained insoluble when the acetic acid was added. It is possible to estimate from Fig. 1 that about $^1/_2$ of the protein is insoluble in acetic acid after a heating period of 33 sec.

Viscosity, optical rotation and rate of digestion by trypsin show significant differences between native BSA and BSA recovered from heat denaturation. The sedimentation pattern of the material designated recovered-BSA is shown in Fig. 2B. The protein had been heated for 15 sec. at 100°, and the recovered material was completely soluble in water. The viscosity of recovered-BSA was found to be slightly greater than that of the native protein. Results are shown in Fig. 3 along with simi-

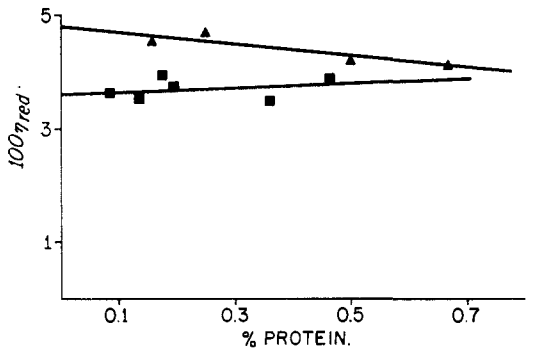

Fig. 3.—Reduced viscosity of native and recovered-BSA: ■, native BSA; ▲, recovered-BSA. The solvent is 0.05 *M* sodium acetate, 0.05 *M* acetic acid, 1.0 *M* sodium chloride, *p*H 4.7.

lar measurements for the viscosity of native BSA in the same acetate buffer. The optical rotation of recovered-BSA was found to be $-59.0 \pm 1.1°$ in acetate buffer *p*H 4.7, while that for native BSA was $-62.0 \pm 0.7°$. Digestion by trypsin was more rapid for recovered-BSA than for native BSA as shown in Fig. 4.

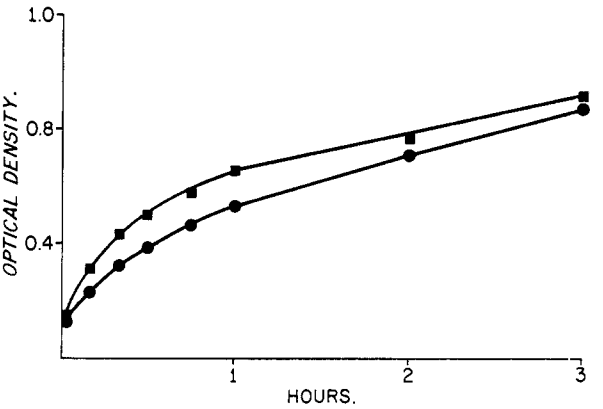

Fig. 4.—Rate of tryptic digestion of native and recovered-BSA as followed by the optical density at 278 mμ of TCA soluble material produced: ●, native BSA; ■, recovered-BSA.

Discussion

The ultracentrifuge patterns shown in Fig. 2 show that the loss of solubility in acetic acid of the heat coagulated BSA is accompanied by the appearance of faster sedimenting components. A heavy component, which appears to be a dimer, is apparent in Fig. 2B. The broadening and skewing of the main peak in Fig. 2C is an indication that there may be several components present.

The appearance of these heavy components is in keeping with the postulation of polymerization by means of disulfide exchange for BSA during heat denaturation[10] and during urea denaturation.[11] The participation of sulfhydryl groups in disulfide exchange has been demonstrated for model compounds by Ryle and Sanger[12] and Fava, Iliceto and Camera.[13] The reaction may be expressed by

$$R_a\text{—SH} + R_b\text{—S—S—}R_b \longrightarrow R_b\text{—SH} + R_a\text{—S—S—}R_b$$

Thus a free sulfhydryl group would participate as a catalytic intermediate.

Although a disulfide exchange through a sulfhydryl intermediate may explain the mechanism of the polymerization of heat denatured BSA, it does not fully explain the *p*H dependence observed. A more complete picture of the reaction, which occurs in the solid state, would require consideration of two different influences: (1) the availability of the sulfhydryl group for participation as an intermediate in the disulfide exchange and (2) the effect of electrostatic repulsion on the packing of the protein molecules, whereby the molecules in the solid coagulum may associate closer together when electrostatic repulsion between them is at a minimum (the isoelectric condition). The closer association

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would allow the disulfide exchange to occur more rapidly than if the molecules were forced apart by electrostatic repulsions.

The following sequence may be postulated for the heat denaturation of BSA: (1) hydrogen bonds are ruptured and rearranged, and the protein molecules aggregate and precipitate in the form of a metastable polymer of protein molecules linked by hydrogen and hydrophobic bonds, (2) a 30 sec. lag follows during which no extensive intermolecular disulfide exchange takes place and (3) after 30 sec. a very rapid intermolecular disulfide exchange occurs.

The addition of acetic acid will dissolve and disperse the polymer formed in (1) but will not break the covalent bonds of the polymer formed in (3). Mercuric ions tie up the sulfhydryl group and prevent the formation of the disulfide polymer in (3), thus the coagulum remains soluble in acetic acid. The structure of BSA is such that, when a solution of the material in (1) is lyophilized, the protein molecules are able to return to something similar to the native state.

BSA recovered after 15 sec. heating is obviously different from the native protein. The presence of a small amount of a dimer which was formed during the heating could explain the increase in the frictional properties as were observed with viscosity. It is difficult to say whether or not the dimer would have a lower optical rotation and increased susceptibility to digestion by trypsin. A complete separation of the monomer from the dimer would be necessary to see whether the monomer of recovered-BSA was identical to native BSA.

Markus and Karush[14] have found that one disulfide group of human serum albumin is more susceptible to reduction than are the rest. A single group, more reactive than the rest in BSA, would explain the appearance of the dimer before the rapid disulfide exchange had started and would also explain the frequent occurrence of small amounts of the dimer in preparations of BSA.

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The Crystal Structure of L-Cystine Hydrochloride[1]

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The crystal structure of cystine hydrochloride has been solved by the method of superposition and refined by two-dimensional F_0 and ΔF syntheses. The configuration of the cystinyl group is the same as that in N,N'-diglycyl-L-cystine dihydrate but different from that in hexagonal cystine. Bond lengths and angles are near expected values and the angle between the planes determined by SS'C' and by S'SC is 79.2°.

The importance of disulfide linkages in protein structure is well known and has been further demonstrated in recent work.[3] Since the cystinyl group occurs frequently in proteins and since the S–S interaction makes an important contribution to the vector space representation of proteins, its configuration is important in X-ray diffraction studies and in model building. It is therefore a matter of some importance to examine the configuration of the cystinyl group under a wide range of conditions.

Yakel and Hughes[4] have determined the configuration of the cystinyl group in a tripeptide N,N'-diglycyl-L-cystine dihydrate, and recently Oughton and Harrison have reported the structure of hexagonal cystine.[5] The present work is an investigation of the configuration of L-cystine as it crystallizes from aqueous hydrochloric acid.

Experimental

Crystals of L-cystine hydrochloride, $HOOC-CH(NH_2\cdot HCl)-CH_2-S-S-CH_2-CH(NH_2\cdot HCl)-COOH$, were prepared by allowing a hydrochloric acid solution to evaporate in a desiccator over phosphorus pentoxide. From oscillation and Weissenberg photographs of the needle-like crystals the following unit cell parameters were determined: $a_0 = 18.61$ Å., $b_0 = 5.25$ Å., $c_0 = 7.23$ Å., $\beta = 103.6°$. Systematic extinctions indicate the space group to be Cm, C2 or C2/m. The density observed by flotation is 1.520 g. cm.$^{-1}$, calculated, 1.515 g. cm.$^{-3}$ assuming 2 molecules per unit cell.

The intensities of the X-ray reflections were measured from unidimensionally integrated Weissenberg photographs with a microdensitometer tracing at right angles to the direction of integration by the camera. The areas under the peaks of the photometric tracings were measured with a planimeter and were assumed to be proportional to the integrated intensities.[6] Results indicate a precision of 2–3% in F_{rel} measured by this method.[7]

Determination of the Structure

The space groups Cm, C2 and C2/m cannot be distinguished by the systematic extinctions of the X-ray reflections. Since these three space groups have at least fourfold general positions, the two molecules of L-cystine hydrochloride must lie in special positions and have some symmetry element of the space group. Since the molecule of L-cystine cannot have symmetry m or i, the only one of the three space groups compatible with possible molecular symmetry is C2.[8] This agrees with that found by Srinivasan[9] and by Corsmit, Schuyff and Feil,[10] and is also the same as was found for N,N'-

(1) Presented at the 4th International Congress of the International Union of Crystallography, Montreal, Canada, July, 1957.

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