The Reaction of Sulphite Ions with an Intermolecular Disulphide Bond in Bovine Serum Non-Mercaptalbumin

By C. McARDLE

Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria

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1. The reaction of SO_3^{2-} ions with bovine serum non-mercaptalbumin was studied at physiological pH. 2. The presence of an intermolecular disulphide bond between the protein and one of two small molecules is confirmed. 3. The bonds are readily cleaved according to the equation:

but the additional presence of 2M-urea is necessary to separate the small molecules from the protein. 4. The points of elution of the small molecules from a column of Sephadex G-25 correspond to those of glutathione and cysteine.

The reaction between SO_3^{2-} ions and compounds containing disulphide bonds has been the subject of much study:

The reaction with simple compounds is reversible, but will go to completion under conditions where the concentration of RS^- is small, such as at pH above 7 (Stricks & Kolthoff, 1951; Cecil & McPhee, 1955). Disulphide bonds in proteins show considerable differences in reactivity. Inter-chain bonds tend to react like those in simple compounds, whereas intra-chain bonds frequently require a denaturing agent or a heavy-metal reagent to be present before any reaction is observed (Kolthoff, Anastasi & Tan, 1958; Cecil & Loening, 1960; Cecil & Wake, 1962).

There is little information on the reactivity towards SO_3^{2-} ions of disulphide bonds between small molecules and proteins. Such bonds are believed to exist in the serum albumins. Hughes (1947, 1949) has shown that the fractional SH content frequently associated with these proteins is due to the presence of two components. The first contains an SH group and is termed mercaptalbumin, and the second has no SH group and is termed non-mercaptalbumin. The molecules are similar apart from this (Hughes & Dintzis, 1964; Spahr & Edsall, 1964). King (1961) has examined the products of performic acid oxidation of human non-mercaptalbumin and suggested that each molecule is linked by a disulphide bond to glutathione or cysteine. Support for this is obtained from the work of Kolthoff, Matsuoka, Tan & Shore (1965), who investigated a number of whole bovine

serum albumins containing various proportions of mercaptalbumin and non-mercaptalbumin. They found that the sum of the initial SH content and the disulphide bonds that were reactive towards sulphite alone approximated to unity for each protein.

The present paper reports a study to confirm the presence of an intermolecular disulphide bond in bovine serum non-mercaptalbumin, to find out whether the protein environment influences its reactivity to SO_3^{2-} ions and to examine the products of the reaction.

MATERIALS AND METHODS

Bovine serum albumin. Two samples of bovine serum albumin were obtained in the freeze-dried form (Koch-Light Laboratories Ltd., Colnbrook, Bucks.). For convenience these are termed BSA 1 and BSA 2. Stock solutions, which were 0.1–0.3 mM, were prepared after exhaustive dialysis against 0.15 M-NaCl.

p-Chloromercuribenzoic acid. The acid was obtained from Koch-Light Laboratories Ltd. Solutions were prepared by dissolving the PCMB* in a minimum quantity of ethanolic NaOH and making up to the required volume with water. A sample of homocysteine (Puriss grade; Koch-Light Laboratories Ltd.) gave a titre of 0.99 ± 0.02 SH group/molecule with these solutions.

All other chemicals were A.R. grade and were used without further purification.

Apparatus. The SH contents of the proteins were measured by amperometric titration with a Cambridge general-purpose polarograph and a dropping-mercury electrode. The characteristics of the capillary were: m , 2.4 mg./sec.; t , 4.0 sec.; h , 61 cm. A saturated-calomel

* Abbreviation: PCMB, p-chloromercuribenzoic acid.

electrode was connected to the cell, of capacity 2 ml., through a saturated-KCl-agar bridge. Potentials are expressed relative to this electrode.

Oxygen was removed from the solutions with hydrogen (Industrial Gases Ltd., Apapa, Nigeria). Frothing of the protein solution was prevented by the addition of a drop of octan-2-ol.

Reaction of the albumins with sulphite. The stock solutions were exhaustively dialysed against 0.05 M- Na_2SO_3 , or 0.05 M- Na_2SO_3 -2 M-urea, in a base electrolyte containing 0.15 M-NaCl in phosphate buffer, prepared by adjusting 0.5 M- KH_2PO_4 to pH 7.4 with 0.5 M- Na_2HPO_4 and diluting fivefold. The reagents were removed by dialysis against 0.15 M-NaCl.

Measurement of thiol groups. Current-voltage and titration measurements were made on 2 ml. of 0.03–0.06 mM-albumin solutions in the base electrolyte. The measurements were repeated in the presence of 8 M-urea to detect any masked SH groups.

Measurement of reactive disulphide bonds. Reactive disulphide bonds are defined as those rapidly cleaved by sulphite alone at physiological pH and room temperature.

A solution of 0.03–0.06 mM-albumin was prepared in 0.05 M- Na_2SO_3 and the base electrolyte. Immediately after preparation 2 ml. of this solution was placed in the polarographic cell and the temperature was lowered to 0° with an ice bath. After 15 min. current-voltage and titration measurements were made on this solution. Any difference between the SH titre of the protein in sulphite and its original titre is considered to be a measure of the number of reactive disulphide bonds.

The validity of this statement was confirmed by preparing the mixture at 0°. No measurable reaction was observed over a period of 1 hr. Thus any increase in titre must be due to a reaction that is complete before the temperature is lowered, and not to the addition of the mercurial forcing the equilibrium reaction to completion by removal of mercaptide ions.

Separation and estimation of the reaction products. This was accomplished by passing 5 ml. of 0.3 mM-albumin in 0.05 M- Na_2SO_3 -2 M-urea in the base electrolyte through a column (48 cm. $\times$ 1.8 cm.) of Sephadex G-25 equilibrated with the same solvent. The flow rate was 60 ml./hr. and the eluate was collected in 5 ml. fractions. The emergence of the albumin was detected by its extinction at 280 m μ . The protein was collected, dialysed exhaustively against 0.15 M-NaCl, made up to 50 ml. and then estimated as described below. The contents of the remaining tubes were each transferred to 10 ml. standard flasks and made up to this volume with the base electrolyte, and 2 ml. portions were titrated with a standard PCMB solution. Thus the liberated thiols were detected and estimated simultaneously. The number of equivalents of SH liberated/mole of albumin was calculated.

Estimation of protein concentration. The micro-Kjeldahl method, with a nitrogen-protein conversion factor 6.25, was used.

RESULTS

The current-voltage curves for the reaction between PCMB and bovine serum albumin are shown in Fig. 1 and a typical titration in Fig. 2. The applied voltage was -0.50 v. The corre-

sponding curves for the reaction in the presence of SO_3^{2-} ions are shown in Fig. 3 and typical titrations in Fig. 4. The applied voltage was -0.60 v. The results of the titrations are shown in Table 1, and are based on a molecular weight of 66 000 for albumin (Spahr & Edsall, 1964).

Original proteins. Both proteins were found to be devoid of SH groups and can be classed as non-mercaptalbumins. No masked groups were detected in 8 M-urea, in which the albumins are unfolded (Imahori, 1960). Titres of about 1 SH

Fig. 1. Current-voltage curve of PCMB in the presence of bovine non-mercaptalbumin: (1) 2 ml. of 0.06 mM-BSA 1 in 0.15 M-NaCl in phosphate buffer, pH 7.4; (2) plus 0.040 ml. of 1.4 mM-PCMB, showing the free PCMB wave; this corresponds to point A in Fig. 2.

Fig. 2. Amperometric titration of bovine non-mercaptalbumin with 1.4 mM-PCMB at -0.50 v: 2 ml. of 0.06 mM-BSA 1 in 0.15 M-NaCl in phosphate buffer, pH 7.4.

Fig. 3. Current-voltage curve of PCMB in the presence of bovine non-mercaptalbumin and sulphite at 0°: (1) 2 ml. of 0.03M-BSA 1 in 0.05M- Na_2SO_3 -0.15M-NaCl in phosphate buffer, pH 7.4; (2) plus 0.080 ml. of 1.4mM-PCMB, showing the free PCMB wave; this corresponds to point B in Fig. 4.

Fig. 4. Amperometric titration of bovine non-mercaptalbumin with 1.4mM-PCMB at 0°, in the presence of sulphite, at -0.60 v: (1) 2 ml. of 0.03M-BSA 1 in 0.05M- Na_2SO_3 -0.15M-NaCl in phosphate buffer, pH 7.4; the end point corresponds to 1.03 SH groups/molecule; (2) a similar titration of BSA 1 after exhaustive dialysis against 0.05M- Na_2SO_3 -2M-urea.

group/molecule were obtained in the presence of SO_3^{2-} ions. This is in accordance with the work of Kolthoff *et al.* (1965) and implies cleavage of a reactive disulphide bond.

Proteins after dialysis against 0.05M-sodium sulphite. Repetition of the previous studies showed little alteration in the various titres. Thus dialysis fails to separate the products of the reaction. The reactive disulphide bond is presumably re-formed during the removal of the SO_3^{2-} ions.

Proteins after dialysis against 0.05M-sodium sulphite-2M-urea. This treatment did not affect the SH content of the proteins but did lead to loss of the reactive disulphide bond. Two equations can be written for the reaction between albumin and SO_3^{2-} ions:

The failure to detect any SH in the albumin after the reaction excludes eqn. (1) unless there is oxidative union of two SH groups to form a disulphide bond, or an intermolecular or intramolecular SH-S-S interchange reaction that transfers the SH group to the interior of the molecule. Since Kolthoff & Anastasi (1958) have shown that oxidative union does not occur in dilute urea solution, and there was no evidence for a masked SH group (Table 1), eqn. (2) is indicated.

Separation and estimation of the reaction products. The above equation was shown to be correct by passing the mixture through a Sephadex G-25 column and testing for the presence of thiols by amperometric titration at -0.60 v. Two fractions were found (Fig. 5) that contained a total of about 1 equiv. of SH/mole of albumin (Table 2). A control experiment showed that the points of

Table 1. *Thiol and reactive disulphide bond contents of bovine non-mercaptalbumin*

Experimental details and the definition of reactive disulphide bond are given in the text. Results are given as the means $\pm$ S.E.M. of four determinations.

	SH or S-S content (equiv./mole of protein)		
	SH	SH in 8M-urea	Reactive S-S bonds
(a) Original proteins:			
BSA 1	0	0	1.03 $\pm$ 0.01
BSA 2	0	0	1.10 $\pm$ 0.02
(b) Proteins after dialysis against 0.05M- Na_2SO_3 :			
BSA 1	0	0	1.04 $\pm$ 0.02
BSA 2	0	0	1.06 $\pm$ 0.01
(c) Proteins after dialysis against 0.05M- Na_2SO_3 -2M-urea:			
BSA 1	0	0	0
BSA 2	0	0	0

Fig. 5. Separation of the products obtained by cleavage of bovine non-mercaptalbumin with sulphite in the presence of 2M-urea. A sample (5 ml.) of 0.3 mM-BSA 1 in 0.05 M- Na_2SO_3 -2 M-urea-0.15 M-NaCl in phosphate buffer, pH 7.4, was passed through a column (48 cm. $\times$ 1.8 cm.) of Sephadex G-25 equilibrated with the same solvent, and the eluent was collected in 5 ml. fractions. ○, Emergence of the protein as measured by its extinction at 280 m μ ; ●, emergence of the simple thiols as measured by amperometric titration with PCMB. The reaction conditions are described in the text. Fractions I and II correspond to the points of elution of glutathione and cysteine respectively.

Table 2. *Estimation of the low-molecular-weight thiols released by the reduction of bovine non-mercaptalbumin with sulphite in 2M-urea*

The number of equivalents of SH in fractions I and II/mole. of protein was estimated as described in the text. Results are given as the means $\pm$ S.E.M. of four determinations.

	SH content (equiv./mole)	
	Fraction I	Fraction II
BSA 1	0.30 $\pm$ 0.02	0.68 $\pm$ 0.02
BSA 2	0.46 $\pm$ 0.01	0.64 $\pm$ 0.02

elution of the fractions corresponded to those of glutathione and cysteine.

DISCUSSION

This study establishes the presence of a single intermolecular disulphide bond in bovine serum non-mercaptalbumin. Chromatography of the modified protein on a column of Sephadex G-25, which acts as a molecular sieve (Porath & Flodin, 1959), shows that it is a bond between the protein and one of two low-molecular-weight compounds. Although these compounds were not subjected to detailed study, the fact that they behave in a similar manner to glutathione and cysteine is in accordance with King's (1961) demonstration that cysteic acid, glycine and glutamic acid are obtained,

in proportions that indicate the presence of this peptide and thiol, after performic acid oxidation of human non-mercaptalbumin and hydrolysis of the non-protein products.

The ease of fission of the disulphide bond is comparable with that of simple disulphides and inter-chain protein bonds. That separation of the low-molecular-weight compounds from the protein is only obtained when urea is present is ascribed to non-covalent bonding. The low concentration of urea used is unlikely to effect any measurable change in the secondary structure of the protein (cf. Callaghan & Martin, 1962), but evidence has been obtained (G. Franglen & C. McArdle, unpublished work) that it can cause a marked weakening of tertiary bonds. Thiol-reducing agents, unlike sulphite, appear to be able to cause fission of both types of bonds (Markus & Karush, 1957; Katchalski, Benjamin & Gross, 1957; Hartley, Peterson & Sober, 1962).

The equation for the reaction shows that the SO_3^{2-} ions attack a half-cystine residue that is part of the polypeptide chain, and not the small molecules. Fava & Iliceto (1958) examined the rate of exchange of sulphite in various organic thio-sulphates by radioactive sulphite and found that an approach 180° to the S-S bond was favoured. It is proposed that the small molecules become masked by surrounding amino acid residues when attached to the protein and that this angle of approach is hindered.

The failure of many workers (Thompson, 1954, 1958; White, Shields & Robbins, 1955; Biserte, 1959; Ikenaka, 1960) to detect the small molecules, by using various methods of end-group analysis, is readily explained if they are masked by the protein. The successful study of King (1961) was made on an albumin that had previously been subjected to countercurrent distribution (King, Yphantis & Craig, 1960). The solvent system brought about dimerization of the mercaptalbumin fraction through the oxidative union of the SH groups to give an intermolecular disulphide bond. This reaction only occurs after unfolding the protein in the neighbourhood of the SH group (Kolthoff & Anastasi, 1958). The non-mercaptalbumin fraction, which was the subject of King's (1961) study, did not dimerize but presumably did unfold, and was thus made amenable to study by end-group analysis.

Finally, it is concluded that the only difference between mercaptalbumin and non-mercaptalbumin is that the latter has its SH group linked by a disulphide bond to a small molecule, probably cysteine or glutathione. The present study was made on samples of albumin that had been prepared by the Cohn fractionation technique. That they were both non-mercaptalbumins was fortuitous,

since the method normally gives a mixture of mercaptalbumin and non-mercaptalbumin (Hughes, 1947; Benesch, Lardy & Benesch, 1955; Simpson & Saroff, 1958). The ease of reaction of the albumin SH group with simple disulphides has been noted by a number of workers (Klotz, Ayers, Ho, Horowitz & Heiney, 1958; Ellman, 1959; King, 1961) as well as in these Laboratories. It is likely that the final quantity of non-mercaptalbumin in a sample of the protein will depend primarily on the amount of free disulphide in its environment before isolation.

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