The Reactions of Inter- and Intra-Chain Disulphide Bonds in Proteins with Sulphite

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Disulphide bonds react with sulphite according to the equation:

The reaction is strictly reversible and will go to completion only under conditions where the concentration of RS^- is small, as for instance at pH 7 (Stricks & Kolthoff, 1951; Cecil & McPhee, 1955).

Insulin has two disulphide bonds linking the A and B chains and a third intra-chain bond linking residues 6 and 11 (referred to below as the 6-11 intra-chain bond) on the A chain (Ryle, Sanger, Smith & Kitai, 1955). Cecil & Loening (1960) studied the reaction of these disulphide bonds with sulphite. They found that the two inter-chain bonds react with sulphite at pH 7 causing the insulin to split into its component A and B chains. The 6-11 intra-chain bond does not react with sulphite alone but it will react with sulphite in the presence of phenylmercuric hydroxide. Partial reaction occurs in the presence of urea or guanidine.

The explanation suggested by Cecil & Loening for this difference in the reactivity of the inter- and intra-chain disulphide bonds was that the structure of the 6-11 intra-chain ring is stabilized by hydrogen bonds (or other non-covalent bonds) rather than by the disulphide bond. This would have the effect of shifting the equilibrium of the reaction with sulphite to the left since the ring would have no tendency to open when the disulphide bond is split. Phenylmercuric hydroxide would block the reverse reaction by combining with the thiol formed. Urea and guanidine would reduce the stability of the 6-11 intra-chain ring by breaking the non-covalent bonds. In these cases the disulphide bond would be expected to react.

Bovine serum albumin has 17 disulphide bonds and these appear to behave similarly to the 6-11 intra-chain bond in insulin. Kolthoff, Anastasi & Tan (1958, 1959) found that there was no appreciable reaction with sulphite alone but that all 17 bonds reacted with sulphite in the presence of mercuric chloride. In the presence of guanidine 12-13 bonds reacted with sulphite. Hunter & McDuffie (1959) have shown that the molecular

weight of bovine serum albumin is unchanged by reduction of all the disulphide bonds. The molecule must therefore consist of a single peptide chain and the disulphide bonds must all be intra-chain.

This paper describes the reaction with sulphite of a number of proteins known to contain disulphide bonds, with a view to determining to what extent any observed differences in reactivity can be ascribed to the presence of intra-chain bonds.

MATERIALS

Oxytocin. A sample was kindly donated by Sandoz Products Ltd., Pharmaceutical Division, 21 Great Castle Street, London, W. 1.

Ribonuclease. Two samples (batch nos. R31B-203 and R80B-202) were purchased from Sigma Chemical Co., St Louis 13, Mi., U.S.A. A sample, which had been purified chromatographically, was kindly supplied to us by Dr W. H. Stein and Dr A. M. Crestfield. Solutions were made up in water and concentrations were estimated from the absorption at 278 m μ . Sela, Anfinsen & Harrington (1957) give ϵ 9800.

α -Chymotrypsinogen. A sample ('A' grade, lot no. 105642) was purchased from the California Corp. for Biochemical Research, Los Angeles 63, U.S.A. Solutions were made up in mM-HCl and concentrations were estimated from the absorption at 282 m μ . Wilcox, Cohen & Tan (1957) give $E_{1\%}^{1\text{cm}}$ 20.0.

α -Chymotrypsin. Crystalline chymotrypsin (lot no. 957) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Solutions were prepared in the same way as with chymotrypsinogen, the same extinction coefficient being used (Chervenka, 1957).

Trypsinogen. A sample ('A' grade, lot. no 23089) was purchased from the California Corp. for Biochemical Research. It contained 50% of $MgSO_4$. Solutions were made up in mM-HCl and dialysed overnight against mM-HCl. Concentrations were estimated from the absorption at 280 m μ . Davie & Neurath (1955) give $E_{1\%}^{1\text{cm}}$ 13.9.

Trypsin. A sample (lot no. 2500) was purchased from Armour Pharmaceutical Co. Ltd. Solutions were made up in mM-HCl. Concentrations were estimated from the absorption at 280 m μ . Davie & Neurath (1955) give $E_{1\%}^{1\text{cm}}$ 14.4.

Lysozyme. Crystalline lysozyme (lot no. DH 2451) was purchased from Armour Pharmaceutical Co. Ltd. Solutions were made up in water. Concentrations were estimated from the absorption at 280 m μ in 20 mM-HCl. Glazer (1959) gives $E_{1\%}^{1\text{cm}}$ 27.3.

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Insulin. Crystalline ox insulin was purchased from Boots Pure Drug Co. Ltd., Island Street, Nottingham. The moisture content was found to be 9.9% and solutions were made up in 10 mM-HCl by weight.

Guanidine chloride. Stock solutions, approximately 7M, were prepared by neutralizing solid, reagent-grade, guanidine carbonate with conc. HCl. The solutions were boiled to eliminate CO_2 .

METHODS

All the reactions with Na_2SO_3 were carried out at 37° under N_2 in a polarographic cell. The concentrations of SH formed during the reactions were estimated by amperometric titration with phenylmercuric hydroxide or HgCl_2 in the same cell (Allison & Cecil, 1958; Cecil & Loening, 1960). The apparatus was the same as that used by Cecil & Snow (1962).

The reactions with sulphite alone were carried out at pH 6.5–7.0 for the reasons given by Cecil & McPhee (1955). The protein concentrations were approximately 0.1 mM. The sulphite concentration was arranged to give an excess of 200–300 moles/disulphide bond. The pH of the reaction mixtures was adjusted by the addition of dil. HCl (the pK_a of H_2SO_3 is 6.8 under these conditions; Cecil & McPhee, 1955). The titrations were carried out with 10mM-phenylmercuric hydroxide at -0.45v (versus the saturated calomel electrode). The temperature was lowered to 0° to prevent the reaction 'following' the addition of the heavy-metal reagent. If a significant amount of SH was present after reaction with sulphite alone a separate titration was carried out in the absence of sulphite to determine the amount of SH present initially. This was done at pH 9.0 in the presence of 0.05M- $\text{Na}_2\text{B}_4\text{O}_7$, 0.05M-HCl and a 300–400 molar excess of sodium dodecyl sulphate (Allison & Cecil, 1958).

The total disulphide content was measured by reaction with sulphite (200–300 moles/disulphide bond) in the presence of 2.5–3.0M-guanidine. The titrations were carried out at the same temperature as the reaction by using 10 mM- HgCl_2 at -0.3v . In the presence of guanidine, HgCl_2 reacts to form mercaptides of the type $(\text{RS})_2\text{Hg}$ (Cecil & McPhee, 1959). These reactions were always repeated in the presence of an excess of HgCl_2 in case there had been a loss of SH due to oxidation (Allison & Cecil, 1958). The first titration is necessary to determine the value of the residual current. The differences in titre obtained in the presence and absence of HgCl_2 varied from 0 to 10%.

RESULTS

Oxytocin

The molecular weight is 980 and there is one disulphide bond per molecule which forms part of the ring structure (Tuppy & Michl, 1953; du Vigneaud, Ressler & Trippett, 1953). The solution supplied was stated to contain 1 mg./ml. and the concentration was taken as 1.02 mM.

After reaction with a 200 molar excess of sulphite for 20 min. there was a liberation of 1.1 SH groups/molecule. Although this result is rather high there is no doubt that the disulphide bond in oxytocin reacts with sulphite.

The oxytocin ring contains the same number of amino acid residues as the 6-11 intra-chain ring in insulin. The ease of reaction with sulphite would suggest, however, that the oxytocin ring is less stable than the 6-11 intra-chain ring of insulin.

Ribonuclease

Ribonuclease has a molecular weight of 13 700. It consists of a single peptide chain containing four disulphide bonds. These form internal rings varying in size from 8 to 59 residues (Spackman, Stein & Moore, 1960).

Reaction with a 800 molar excess of sulphite (i.e. 200 moles/disulphide bond) at pH 6.9 gave a liberation of 0.5 SH/molecule after reaction periods of 2 hr. Longer reaction times of up to 4 hr. gave the same result, and so the figure of 0.5 can be taken as the maximum number of disulphide bonds reacting with sulphite alone.

The work on the 6-11 intra-chain disulphide bond in insulin was based mainly on its reaction with sulphite in the presence of excess of phenylmercuric hydroxide. This reaction proved to be very slow for ribonuclease, only 1.5 bonds reacting after 3 hr. Longer reaction times than this were undesirable in view of Cecil & Snow's (1962) observation that phenylmercuric hydroxide reacts slowly with sulphite to give diphenylmercury and Hg^{2+} ions.

Fig. 1. Rates of reaction of the disulphide groups of ribonuclease with sulphite in the presence of HgCl_2 and either 6M-urea (A) or 2.2M-guanidine (B) at 37°. The reactions were followed by observing the decrease with time in height of the polarographic wave of Hg^{2+} ions (Cecil & Snow, 1962).

Table 1. *Reaction of the disulphide bonds of different proteins with sulphite*

The number of disulphide bonds reacting with sulphite both alone and in the presence of guanidine plus HgCl_2 are given. All reactions were carried out at pH 6.5–7.0 unless otherwise stated. With the exception of lysozyme, which required $1\frac{1}{2}$ hr., all reactions with sulphite + guanidine were complete in 30 min. or less.

Substance	Reaction with sulphite alone		Reaction with sulphite plus guanidine and HgCl_2 (S·S bonds/mole)
	Free SH groups before reaction with sulphite	S·S bonds/mole	
Oxytocin	—	1.1	—
Ribonuclease	0	0.5	3.95
Insulin	0	1.9*	3.05 (pH 6–9)
Lysozyme	—	0.1	3.9 (pH 6.8 and 9)
α -Chymotrypsinogen	0.06	0.8	4.7
α -Chymotrypsin	0.06	3.1	4.7
Trypsinogen	—	5.1	5.9
Trypsin	0	5.2	5.9
Bovine serum albumin†	0.7	0	17

* Cecil & Loening (1960).

† Kolthoff, Anastasi & Tan (1958).

This difficulty can be overcome by using HgCl_2 provided that urea or guanidine is present. This is necessary to ensure that only the dimercaptide, $(\text{RS})_2\text{Hg}$, is formed and not a mixture of that and RSHgCl . The relative rates of reaction in 6M-urea and 2.2M-guanidine at pH 7 are shown in Fig. 1. Since the reaction in the presence of guanidine was significantly faster it was used throughout the rest of the work for estimating total disulphide.

The two samples of ribonuclease obtained from Sigma Chemical Co., which were used for most of the work, gave low values for the total number of disulphide bonds per molecule, namely 3.4. However, the purified sample provided by Dr W. H. Stein and Dr A. M. Crestfield gave a value of 3.95. In view of this it was assumed that the Sigma material contained an impurity and the results were corrected accordingly.

The extent of reaction with sulphite alone represents only 12% of the total disulphide present. To this extent all four disulphide bonds in ribonuclease are behaving similarly to the 6–11 intra-chain bond in insulin.

The reaction in the presence of phenylmercuric hydroxide is in fact slower than that with insulin and so some, at least, of the ribonuclease disulphides must be more stable.

The fact that the number of amino acid residues in the internal rings varied from 34 to 8 suggests that this is not a major factor in determining the stability of the ring.

Insulin

The two criteria required are the extent of reaction with sulphite alone and the total disulphide content. The results with ribonuclease suggest that the best general method for total disulphide content is reaction with sulphite in the presence of guanidine and HgCl_2 . Experiments

were therefore made with insulin to confirm that the 6–11 intra-chain bond reacted quantitatively under these conditions. Reactions were carried out in the range pH 6–9 with a 800 molar excess of sulphite. The reaction mixtures were titrated with HgCl_2 , as described under Methods, after 30 min. There was no significant difference over the pH range used and a mean value of 3.05 disulphide groups/molecule was obtained.

This method was therefore used with the remaining proteins and the results are summarized in Table 1.

Lysozyme

The molecular weight is 14 800 (Warner, 1954) and the molecule consists of a single peptide chain (Fraenkel-Conrat, Mohammed, Ducay & Meedom, 1951). Estimates of the number of disulphide bonds vary between 4 (Dose & Caputo, 1956; Jolles, Bernier, Jaurequi & Jolles, 1960) and 5 (Thompson, 1955; Anfinsen, 1958; Glazer & Smith, 1961).

The results, given in Table 1, show that less than 0.1 disulphide bond reacts with sulphite alone and 3.9 react in the presence of guanidine. These disulphides are unusually stable, a reaction time of $1\frac{1}{2}$ hr. being required. No further reaction could be detected after this time.

It appears therefore that the figure of 4 disulphide bonds/molecule is correct.

α -Chymotrypsinogen

The molecular weight is 25 000 and there are five intra-chain disulphide bonds per molecule (see review by Neurath, 1957). One of the cystine residues is *N*-terminal (Bettelheim, 1955).

The number of disulphide bonds reacting with sulphite alone was 0.8, and in the presence of

guanidine 4·7. Pechère, Dixon, Maybury & Neurath (1958) used the reaction with sulphite in the presence of Cu^{2+} ions and obtained values of 4·5–5·0 disulphide bonds/molecule.

It is possible that the bond which reacts with sulphite alone is one involving the *N*-terminal cysteine, since the molecule might be expected to be most flexible in that region.

α -Chymotrypsin

α -Chymotrypsin is produced by the slow tryptic activation of chymotrypsinogen. During the course of the activation the peptide chain is broken in two places. The molecular weight was taken as 24 500.

Meedon (1956) has shown that, on oxidation with performic acid, chymotrypsin splits into three peptide chains designated A, B and C. The number of half-cystines in these chains has been found by Meedon (1956) and Hartley (1958) to be A = 1, B = 6 and C = 3. This gives three possible structures, as illustrated in Fig. 2. Structure I has 2 inter- and 3 intra-chain disulphide bonds, structure II has 3 inter- and 2 intra-chain bonds and structure III has 4 inter- and 1 intra-chain bonds.

The results in Table 1 show that 3·1 disulphide bonds react with sulphite alone as compared with a total of 4·7. If it is assumed that the 3·1 bonds reacting with sulphite are all inter-chain this

favours structure II. On the other hand, it is possible that one of these bonds is intra-chain as in chymotrypsinogen, and that structure I is correct. On this evidence, therefore, only structure III can be eliminated.

Trypsinogen

The molecular weight is 24 000 and there are six disulphide bonds per molecule. Since only one *N*- and one *C*-terminal group have been identified these bonds must all be intra-chain (Neurath, 1957).

The results in Table 1 show that five out of six of these intra-chain bonds react with sulphite alone. This behaviour differs markedly from that of the other proteins so far discussed.

Pechère *et al.* (1958) studied the products of reaction of both chymotrypsinogen and trypsinogen with sulphite and Cu^{2+} ions. This procedure was shown to split all the disulphide bonds. The specific optical rotation of chymotrypsinogen changed from $-70\cdot5^\circ$ to $-81\cdot2^\circ$ during the course of the reaction, whereas trypsinogen changed from $-36\cdot2^\circ$ to $-86\cdot7^\circ$. The large change in trypsinogen suggests that a considerable re-orientation of the structure accompanies the splitting of the disulphide bonds. It is possible therefore that the structure of trypsinogen is under some strain, causing the intra-chain bonds to behave more like inter-chain bonds, whereas with chymotrypsinogen there is less strain.

Fig. 2 Possible arrangements of the disulphide bonds in α -chymotrypsin based on the amino acid analyses and the notation of Meedon (1956) and Hartley (1958).

Trypsin

The conversion of trypsinogen into trypsin (molecular weight approximately 23 500) involves the splitting off of one small peptide without any major change in the structure of the protein (Neurath, 1957). It would be expected therefore that the disulphide bonds would react in a similar way to trypsinogen. The results in Table 1 show that this is the case.

DISCUSSION

Cecil & Loening (1960) showed that the 6–11 intra-chain disulphide bond of insulin differed from the two inter-chain bonds in its inability to react with sulphite. The present work has shown that the intra-chain bonds in ribonuclease and lysozyme behave in the same way. The 17 disulphide bonds in bovine serum albumin have also been shown to be intra-chain and not to react with sulphite (Hunter & McDuffie, 1959; Kolthoff *et al.* 1958, 1959).

There is reason to suppose that the explanation proposed by Cecil & Loening for this behaviour is correct. According to them the intra-chain rings are stabilized by hydrogen bonds (or other non-covalent bonds) rather than by the disulphide bonds. If this is so then, if the disulphide bonds are broken and re-formed, one would expect a high probability that the protein is reconstituted in its original form. This has been shown to occur with insulin, ribonuclease and serum albumin.

Dixon & Wardlaw (1960) treated insulin with sulphite and Cu^{2+} ions (Svan, 1957), which converts all three disulphide bonds into *S*-sulphonate. The reaction was then reversed with 2-mercaptoethanol and it was shown by activity measurements and immunological tests that there had been a 1–2% regeneration of the original insulin. Since this process involves not only the re-formation of the intra-chain bond, but also the more random re-formation of the two inter-chain bonds, this yield is not unduly low.

Anfinsen & Haber (1961) and White (1961) reduced the disulphide bonds of ribonuclease with 2-mercaptoethanol in urea. The reduced protein was reoxidized by exposure to air. The reoxidized protein had 80–100% of the original enzymic activity and its properties corresponded closely with original material. It can be argued that, as all the disulphide bonds are intra-chain, the chances of re-covering the original protein are higher than with insulin. Nevertheless, as White has pointed out, there are 105 possible isomers and so it is a convincing demonstration of the preferential stability of the original structure.

Kolthoff, Anastasi & Tan (1960) treated bovine

serum albumin with sulphite in the presence of 4-m-guanidine. This treatment was shown to break 14 out of the 17 disulphide bonds. The reaction was reversed by alternately lowering the pH to 1.5 to remove sulphite as sulphur dioxide and raising it to 5 to allow the reverse reaction to proceed. Provided that the protein concentration was 0.25% or less the original viscosity was restored. This by itself is not proof that the original structure had been re-formed. Nevertheless viscosity provides a sensitive test for molecular size and shape and one can at least assume that a molecule of similar size and shape has been formed.

It appears therefore that the disulphide bonds in ribonuclease, bovine serum albumin and probably lysozyme behave in the same way as the intra-chain bond in insulin and for the same reason. However, the fact that the bond in oxytocin reacts with sulphite shows that these ring systems do not invariably have a stable structure. This is confirmed by the behaviour of the other proteins investigated.

One of the five intra-chain bonds in chymotrypsinogen reacts with sulphite, as do five of the six in trypsinogen and trypsin. Possible reasons why these bonds should differ from the other intra-chain bonds have already been suggested. The difference in behaviour between chymotrypsinogen and chymotrypsin probably represents a change from intra- to inter-chain bonds and is of interest because it throws some light on the structure of chymotrypsin.

One may conclude that intra-chain disulphide bonds commonly, but not invariably, form part of stable ring systems. When the ring systems are stable then the disulphide bonds do not react with sulphite. The size of the ring does not necessarily affect the stability. No instance has yet been observed of an inter-chain bond's failing to react with sulphite though, as in insulin (Cecil & Loening, 1960), there may be differences in the rate of reaction.

The role of disulphide cross-links in protein synthesis has been discussed by Kauzmann (1959) and by Vaughan & Steinberg (1959). It would appear that with proteins of the type of structure found in ribonuclease, lysozyme and serum albumin only two processes are required. The first forms the amino acids into a peptide chain in a predetermined order. The second oxidizes the SH groups of the cysteine residues to form intra-chain disulphide bonds. Any one sequence of amino acids will presumably have a limited number of preferred configurations. The number of the preferred configurations which will permit the oxidation of the SH groups to form intra-chain disulphide bonds will be further limited, possibly to one only as with ribonuclease.

Other mechanisms will presumably be needed for proteins such as insulin, where peptide chains have to be linked together, and with trypsin and trypsinogen where the structure appears to be strained.

The method of estimating total disulphide used in this work, namely reaction with sulphite in the presence of guanidine plus mercuric chloride (also used by Kolthoff *et al.* 1958), is the most convenient of the various methods tried.

SUMMARY

1. The reaction of the disulphide bonds in a number of proteins with sulphite has been studied under various conditions.

2. It has been shown that, whereas the inter-chain disulphide bonds react with sulphite alone, the majority of intra-chain disulphide bonds do not.

3. All the disulphide bonds react with sulphite in the presence of guanidine and mercuric chloride and this reaction was used for the estimation of the total disulphide content of the proteins.

4. These findings have been interpreted in terms of the structure of the proteins concerned.

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