

IV. 5. ON THE ORDER OF DISULFIDE BOND REDUCTION IN RIBONUCLEASE

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I. Introduction

Protein disulfide bonds may be reductively cleaved by a number of SH-containing reagents. Many early observations were made on the gross effects of reduction on proteins and have been adequately reviewed elsewhere (1-4). More recently various studies on the relationship between the extent of reduction of proteins and their biological activity have been reported. Kern (5) found that reduction of one-third of the disulfide bonds in pepsin had little effect on the enzymatic activity, which could only be abolished by cleavage of the remaining disulfide bonds. Liener (6), however, reported complete inactivation of trypsin with only partial reduction. Complete reduction of lysozyme (7, 8) and ribonuclease (RNase) (8, 9) yielded products devoid of enzymatic activity.

The reduction of protein disulfide bonds by SH-containing reagents is apparently specific (8), and therefore it should be ideal for the study of the contribution of disulfide bonds to the physical and catalytic properties of proteins. With recent advances in the knowledge of protein structure, the correlation of observed properties with specific disulfide bonds has become increasingly feasible. Lindley (10) has already obtained evidence that the intrachain disulfide bond of insulin is cleaved preferentially on partial reduction. Since hormonal activity is completely lost on par-

tial reduction, it would appear that this disulfide bond is at least in part responsible for maintaining activity.

The amino acid sequence of RNase is now essentially established (11-15), and the locations of the disulfide bonds have been reported (16, 17). Half-cystine residue No. 1 (counting from the N-terminal end) appears to be joined to half-cystine No. 6, No. 3 to No. 7, No. 2 to No. 8, and No. 4 to No. 5*. This information will ultimately make possible a comparison of the enzymatic properties of homogeneous samples of partially reduced RNase and those of the native protein in structural terms. The problem of purifying partially reduced RNase remains to be solved. Preliminary fractionation studies on partially reduced, carboxymethylated RNase in this laboratory have indicated a high degree of heterogeneity; it has been suggested (18) that the mixed disulfide content of the partially reduced enzyme is largely responsible for this effect.

A prerequisite for the isolation of any product is the development of a suitable means for its assay. A method which may be used for determination of the extent of homogeneity with respect to reduction of specific disulfide bonds in reduced RNase is described in the present paper. A sample of reduced, carboxymethylated RNase is subjected to further reduction under such conditions that complete cleavage of the remaining disulfide bonds should occur, and radioactive iodoacetic acid is employed for carboxymethylation of this product. The resulting completely reduced and carboxymethylated RNase is degraded proteolytically, and, after separation of the peptides, the degree of labeling of each radioactive peptide is taken as a measure of the extent to which the corresponding disulfide bond was intact in the original sample. This approach has been used in a preliminary investigation of the order of disulfide bond reduction by the assay of unfractionated, reduced, carboxymethylated RNase.

II. Methods

1. PARTIAL REDUCTION OF RNASE

Reductions of bovine pancreatic RNase (Armour lot No. 381-059) to various levels (up to 4 SH groups per mole) were carried out as follows: RNase was dissolved to a concentration of 10-12 mg. per ml. in a water solution of thioglycolate (previously adjusted to pH 8.5 with trimethylamine) of such concentration that the final molar ratio of thioglycolate to RNase was 400:1. The reaction was allowed to proceed at room temperature with nitrogen bubbling through the mixture. After the desired

* Due to the possibility of some chemical rearrangement during isolation and characterization of cysteic acid peptides, some uncertainty remains in regard to the 2-8 and 3-7 bonds.

time, the protein was precipitated by addition of ice-cold, acidified acetone and washed with this solvent and with ether as previously described (9). To arrive at a level of reduction for which titration with *p*-chloromercuribenzoate (PCMB) indicated the presence of 2 SH groups per mole of protein, the reaction time was 20 minutes. For the "4 SH level" of reduction, the time was 40 minutes. A level of reduction approximating 6 SH groups per mole was achieved with a reaction time of 25 minutes in the presence of 8 *M* urea; other conditions were as described above. Estimation of reduction levels was routinely made by spectrophotometric titration with PCMB by a modification of the technique of Boyer (19). The exact details of the procedure used are given elsewhere (8).

2. CARBOXYMETHYLATION OF REDUCED RNASE

Iodoacetate reacts with the SH groups that appear on reduction of RNase to yield *S*-carboxymethylcysteine (SCMC) residues. This reaction has been employed routinely to prevent the reformation of disulfide bonds by autoxidation. A molar ratio of 700:1 for iodoacetic acid to protein was used originally in this reaction (9). In subsequent investigations, however, a ratio of 70:1 was found to serve the same purpose and therefore was used throughout the work reported in this paper. Otherwise the procedure was that reported previously. The precipitation of protein at the end of the reaction and its washing were carried out as indicated above.

3. ASSAY FOR SPECIFIC DISULFIDE BONDS IN REDUCED RNASE

Partially reduced, carboxymethylated RNase was subjected to complete reduction. The reaction mixture was 8 *M* with respect to urea and the reaction time was 4.5 hours. Other conditions were as described above for partial reduction. The product was carboxymethylated with iodoacetic acid 1-C¹⁴ with a specific activity of 0.23 mc. per mM. After precipitation and washing the protein was redissolved in water and lyophilized. Five mg. of the dried protein were dissolved in 1 ml. of freshly prepared 0.1 *M* ammonium carbonate—HCl buffer of pH 8.0. The reaction mixture contained 0.005 ml. of a phenol red solution (0.1% in aqueous 20% ethanol) as a control on the pH. Ten μ l. of a trypsin* solution (5 mg. per ml. in water) were added, and the mixture was incubated at 37° for 2 hours. Ten μ l. of a chymotrypsin* solution (5 mg. per ml. in water) were then added and incubation continued for another 2 hours. The digest was lyophilized for 18 hours to ensure removal of sufficient ammonium carbonate to eliminate the salt effect in the subsequent chromatography. The dried sample was redissolved in a small volume of water, and one-half of the final vol-

* Crystallized, Armour.

ume was dried as a small spot on Whatman No. 3 paper. The sample was chromatographed (descending) in the organic phase of butanol-acetic acid-water (4:1:5), prepared on the day of use. The paper was dried at room temperature and cut into two halves. Each half was subjected to electrophoresis in a pyridine-acetic acid buffer of pH 6.5 under toluene by the technique of Michl (20) at 35 volts per cm. for 1.5 hours*. After the papers were dried in a ventilated oven at 80° for 15 minutes, they were dipped in 0.5% ninhydrin in absolute ethanol containing 1 ml. of collidine per 400 ml. of solution. The papers were oven-dried as before to develop the color. Radioautographs of the resulting fingerprints were prepared by exposure of X-ray film to the papers for 72 hours, followed by development of the film under standardized conditions.

For amino acid analysis, the radioautographs were prepared before development of the ninhydrin color. The labeled peptides, which were located by their positions on X-ray film, were excised from the fingerprints and eluted with 6 *N* HCl, in which they were hydrolyzed at 105° for 18 hours in sealed tubes. The hydrolyzates were dried *in vacuo* and chromatographed for 18 hours on Whatman No. 1 paper in the butanol-acetic acid-water system. The papers were dried at room temperature and chromatographed in the second dimension with aqueous 80% pyridine. The papers were dried as before and developed by dipping in an absolute ethanol solution of 0.1% ninhydrin-glacial acetic acid-collidine (15:5:2) followed by oven-drying at 80°.

Approximations of the peptide concentration on the fingerprints and the extent of labeling were made by scanning of the ninhydrin spots and the corresponding spots on film, respectively, with a model 525 densitometer (Photovolt Corporation). Optical density curves were obtained in this way for each spot, and the area under each curve was taken as a measure of the peptide concentration or the extent of its labeling. The ratio of this area for the radioautographic spot to that of the corresponding ninhydrin spot constituted a measure of the specific activity of the peptide under investigation. With knowledge of the amino acid content of the peptide it was possible to assign it a position in the RNase chain and thereby to conclude which of the half-cystine residues was present in the peptide (as SCMC). The specific activity of each peptide was used as an indication of the extent to which the corresponding disulfide bond was present in the original sample of reduced, carboxymethylated RNase.

* A similar procedure has been used in the "fingerprinting" of proteins by Ingram (21) who, however, employed electrophoresis first and chromatographed in the second dimension. A more detailed account of the present method is in preparation by Drs. A. N. Katz and W. J. Dreyer of this laboratory.

III. Results and Discussion

Several factors influenced the choice of proteolytic enzymes for the degradation of reduced RNase. Since the action of trypsin is restricted to peptide bonds involving lysine and arginine, there would be no formation of "families" of closely related peptides with the use of this enzyme. While chymotrypsin is not as ideal in this respect, it was used in combination with trypsin to obtain peptides small enough for convenient chromatographic and electrophoretic separation. All protein samples subjected to the action of these enzymes had been completely reduced prior to the final carboxymethylation and presumably differed only to the extent of

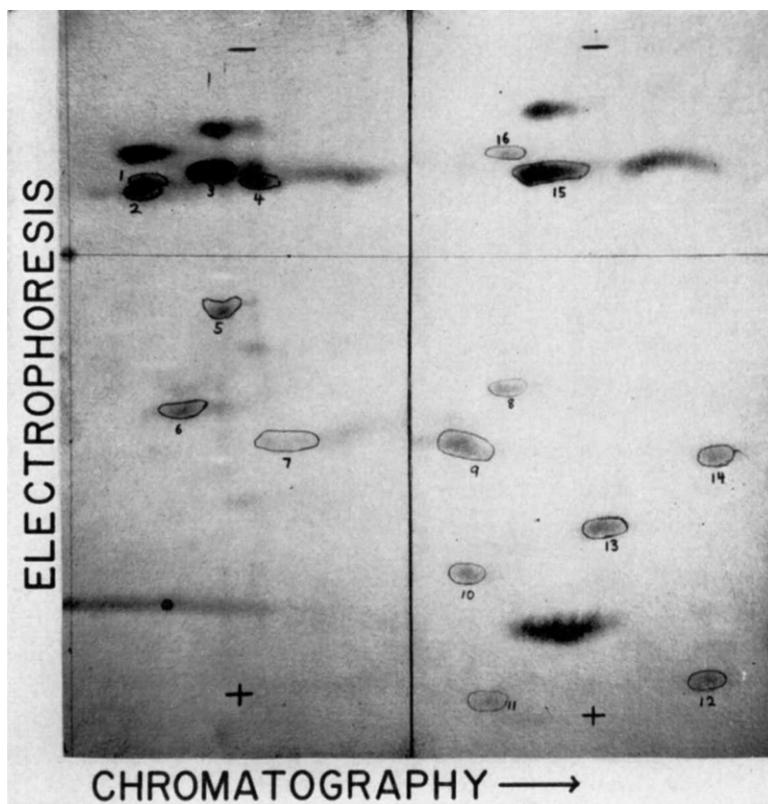


FIG. 1. Two dimensional separation of peptides from a trypsin and chymotrypsin digest of completely reduced, carboxymethylated ribonuclease. When iodoacetic acid $1-C^{14}$ was used for carboxymethylation, the encircled and numbered peptides were found to be labeled.

the labeling of their carboxymethyl groups. Therefore, any unusual cleavages by the proteases employed would be expected to occur in an identical way with all the samples.

The enzymatic digests of these samples, when analyzed two-dimensionally, always yielded the same ninhydrin-positive pattern as shown in Fig. 1. The results obtained thus far in amino acid analysis of the radioactive peptides from such fingerprints are given in Table I, with con-

TABLE I
AMINO ACID ANALYSIS OF LABELED PEPTIDES

Peptide ^a	Amino acid content in addition to SCMC	Half-cystine residue number ^b	Disulfide bond ^c
5	Lys, Asp, Glu, Ser, Ala Val, Leu, His	3	3-7
7	Asp, Glu, Gly, Thr	5	4-5
9	Arg, Asp, Ser, Thr, Leu ^d	6	1-6
12	Lys, Asp, Thr, Pro, Val	2	2-8
14	Tyr, Pro, Asp, Ala	7	3-7

^a The peptides are numbered as shown in Fig. 1.

^b The half-cystine residues are numbered from the N-terminal end of the ribonuclease chain.

^c The disulfide bonds are given as reported previously (14, 15) (see footnote on page 280).

^d Leucine and isoleucine are indistinguishable by the chromatographic technique used (described in text).

clusions as to which SCMC residue is contained by each peptide. From the observed specific activities of these peptides, the percentage of each disulfide bond was calculated for various levels of reduction (Tables II and III) and plotted in Fig. 2. The points for the 1-6 disulfide bond are connected to emphasize its complete disappearance at a level of reduction for which the remaining bonds are clearly in evidence. When the starting material was completely reduced, carboxymethylated RNase, no disulfide bonds were detected by this method. It is not considered possible, from these preliminary results, to arrive at any conclusion regarding the relative liabilities of the 2-8, 3-7, and 4-5 bonds. A correlation of RNase activity loss with reduction is presented in Fig. 3. A comparison of the activity loss with disappearance of disulfide bonds (Fig. 2) strongly suggests that the 1-6 bond is not essential for RNase activity.

Confirmation and extension of this work will be greatly facilitated by purification of partially reduced RNase. The homogeneity of a sample

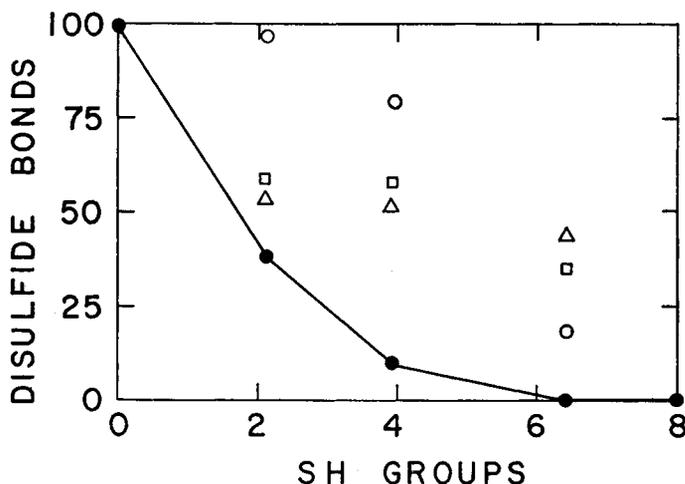


Fig. 2. Disappearance of disulfide bonds in ribonuclease with reduction. The disulfide bond content is expressed as per cent of the specific activity of the corresponding peptide (see Table I), with the specific activity of this peptide for native ribonuclease taken as 100 (see Tables II and III). The reduction levels were determined by titration of reduced ribonuclease with *p*-chloromercuribenzoate. The specific activities of peptides 5 and 14 were averaged to obtain points for the 3-7 disulfide bond. KEY: ●, 1-6 disulfide bond; ○, 4-5 disulfide bond; □, 3-7 disulfide bond; △, 2-8 disulfide bond.

TABLE II
CONCENTRATION AND RADIOACTIVITY MEASUREMENTS OF ISOLATED PEPTIDES

Peptide ^a	SH: ^d	Radioactivity ^b					Concentration ^c				
		0	2.1	3.9	6.4	8.0	0	2.1	3.9	6.4	8.0
5		8.4	5.6	6.0	3.0	0	17	20	24	16	22
7		7.8	7.0	4.1	1.1	0	2.6	2.4	1.7	2.0	2.1
9		22.0	11.0	2.5	0	0	1.7	2.1	1.9	2.2	1.9
12		5.9	3.9	4.4	1.9	0	4.9	5.3	5.6	5.0	5.2
14		7.4	5.0	4.2	3.2	0	6.2	7.5	6.7	6.1	6.4

^a The peptides are numbered as shown in Fig. 1.

^b The figures are proportional to areas under the curves (optical density versus mm.) resulting from photometric scanning of the corresponding radioautographic spots.

^c The figures are proportional to areas under the curves (optical density versus mm.) resulting from photometric scanning of the corresponding ninhydrin-developed spots (see Fig. 1).

^d Sulfhydryl groups per mole of reduced ribonuclease, determined by titration with *p*-chloromercuribenzoate

TABLE III
 SPECIFIC ACTIVITIES OF ISOLATED PEPTIDES

Peptide ^a	SH: ^d	Specific activity ^b					Specific activity (reduced) Specific activity (native) × 100 ^c			
		0	2.1	3.9	6.4	8.0	2.1	3.9	6.4	8.0
5	0.50	0.28	0.25	0.19	0	56	50	38	0	
7	3.0	2.9	2.4	0.55	0	97	80	18	0	
9	13.0	5.2	1.3	0	0	40	10	0	0	
12	1.2	0.74	0.79	0.38	0	62	66	32	0	
14	1.2	0.67	0.63	0.52	0	56	53	43	0	

^a The peptides are numbered as shown in Fig. 1.

^b Specific activity was obtained by dividing the value for radioactivity by that for concentration (Table II) for each peptide.

^c These values were plotted against reduction level (Fig. 2).

^d Sulfhydryl groups per mole of reduced ribonuclease, determined by titration with *p*-chloromercuribenzoate.

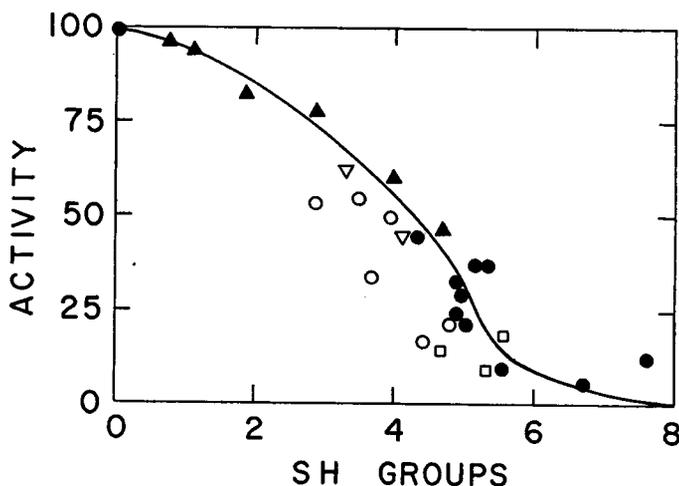


Fig. 3. Activity of ribonuclease at various stages of reduction (expressed as per cent of the specific activity of native ribonuclease) as a function of the number of moles of sulfhydryl per mole of enzyme. Ribonucleic acid was the substrate. Details of the experimental procedures are given elsewhere (9). KEY: ▲, reduction in absence of urea; ●, reduction in 8 *M* urea; □, reoxidation of fully reduced, inactive ribonuclease; ○, reoxidation of samples containing more than six sulfhydryl groups per average molecule; ▽, reoxidation of samples containing about four sulfhydryl groups per average molecule. A curve has been drawn through the black points to indicate the approximate course of reduction. (It is noteworthy that the points representing the reoxidation experiments roughly follow the same curve, indicating a specific rather than random reformation of disulfide bonds.)

of partially reduced protein with respect to the absence of certain disulfide bonds would be indicated by the total absence of labeling in the peptides corresponding to the bonds in question. The technique reported here serves to illustrate the approach that may be used as an assay in future attempts to obtain homogeneous samples of partially reduced proteins.

IV. Summary

A method for following the reduction of specific disulfide bonds in ribonuclease has been outlined. This method has been used for the study of ribonuclease at various levels of reduction. The results indicate that the disulfide bond between half-cystine residues 1 and 6 is the most labile under the present conditions of reduction and that this bond is not essential for ribonuclease activity.

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Discussion

BENESCH: Have you done any nonenzymatic hydrolyses?

WHITE: We have just used trypsin and chymotrypsin for hydrolysis of reduced,

carboxymethylated ribonuclease. Nonenzymatic hydrolysis would be nonspecific, and we would very likely get families of closely related peptides. This would make our job more difficult. Trypsin is especially good for hydrolysis because of its high degree of specificity.

BENESCH: Yes. The only reason why I asked is because in the case of insulin Sanger had to resort to acid hydrolysis because the proximity of the two disulfide bonds on the A chain made this particular region unsusceptible to trypsin.

WHITE: We have no such problem with ribonuclease. With the use of trypsin and chymotrypsin, the result is one half-cystine residue per peptide.

BROWN: Have you tried in your reduction studies to correlate the relationship between the SH values obtained by analysis and that obtained by any of the other titration methods?

WHITE: To what other methods are you referring?

BROWN: I am talking about the SH titration method such as the PCMB one. I wonder whether you tried to reduce one disulfide group or two disulfide groups, treated the protein with iodoacetamide, and checked this result versus a straight titration method for SH. Have you done anything like that?

WHITE: We have compared PCMB titrations after partial reduction of ribonuclease with those after carboxymethylation and complete reduction of the same sample. Thus far the results indicate that the sum of the two is close to the theoretical value of 8.

FRAENKEL-CONRAT: I think the question refers to the specificity of the iodoacetamide reaction.

BROWN: It does.

FRAENKEL-CONRAT: We use both iodoacetic acid and other methods and find them to correspond very closely. In a limited time period at around pH 7 to 8 there is no reaction with groups other than sulfhydryl. So this is as good a method to tag sulfhydryl groups as any.

KAPLAN: Do you have to use urea also to get oxidation of the disulfide?

WHITE: No, that is not necessary. The way we have carried it out is with performic acid, and we do not need urea for that.

KAPLAN: How do you explain this?

WHITE: Thioglycolic acid is apparently specific for disulfide bonds. For complete reduction, urea must be used as a denaturing agent and appears to act by cleaving hydrogen bonds. Performic acid seems to have a sufficiently strong denaturing effect of its own so that urea need not be used with it.