An Unusual Disulfide Bond in Streptococcal Proteinase*

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The proteinase produced by Group A hemolytic streptococci has the unique property of containing only a single half-cystinyl residue per molecule (Liu, Neumann, Elliott, Moore, and Stein (1)). The —SH group of the potential cysteinyl residue is liberated only when the enzyme is activated by reduction. These findings raise the question as to the nature of the linkage in which the half-cystinyl residue participates in the unreduced protein.

The presence of only one half-cystinyl residue is well established (1). Performic acid oxidation followed by acid hydrolysis of the zymogen or of the unreduced enzyme yields only one residue of cysteic acid per molecule and no other strongly acidic, ninhydrin-positive amino acid. After reduction by thiols or sodium borohydride, the presence of a single highly reactive sulphydryl group is readily demonstrated; brief treatment of the reduced protein with iodoacetate, for example, leads to the formation of one residue of S-carboxymethylcysteine per molecule. The reductive step causes no detectable change in molecular weight.

This communication is concerned first with experiments designed to learn whether or not a thioester group is present. Since the results of tests for thioester were negative, evidence was sought for the possible presence of a disulfide bond in which the second sulfur atom is contributed by a mercaptoan other than cysteine.

**EXPERIMENTAL PROCEDURE**

**Materials**—The experiments in this series have been carried out with zymogen since the crystalline protein, as isolated by Elliott (2) from the culture medium, is in a nearly homogeneous form without ion exchange chromatography (1). The crystalline material was dissolved in 0.02 M phosphate buffer, pH 5.0, and subjected to gel filtration on a column of Sephadex G-75 (150 × 2 cm) as described by Liu et al. (1). The resulting protein solution was mixed with an equal volume of glacial acetic acid and decanted by passage through a Sephadex G-25 column (2 × 35 cm) equilibrated and eluted with 50% acetic acid. The solution was subjected to gel filtration on a column of Sephadex G-75 (150 × 2 cm) as described by Liu et al. (1). The resulting protein solution was mixed with an equal volume of glacial acetic acid and decanted by passage through a Sephadex G-25 column (2 × 35 cm) equilibrated and eluted with 50% acetic acid. The solution of acid-denatured zymogen was freeze-dried and stored for further use.

**Urea** was recrystallized by the procedure of Benesch, Lardy, and Benesch (3) and solutions were made up freshly before use. Sodium borohydride, obtained from Metal Hydrides Inc., Beverly, Massachusetts, was stored over CaC₂. Iodoacetic acid was obtained from Matheson, Coleman and Bell. Glutathione (obtained from Schwarz Laboratories, Lot GL 5723) was 100% pure by chromatography and amperometric titration of the —SH groups. S-Acetylglutathione (Nutritional Biochemicals Corporation)

C₅H₁₀O₄N₂S (349.4)

Calculated: C 41.25, H 5.48, N 12.84, S 9.17
Found: C 41.29, H 5.18, N 12.70, S 10.83

gave a major peak on the 50-cm column of the amino acid analyzer at the same position as both oxidized glutathione and aspartic acid (30 ml) and a minor peak representing about 1.6% of the total ninhydrin color, at the glutathione position (34 ml). Hydroxylamine hydrochloride was stated by the suppliers (Amend Drug & Chemical Company, Inc.) to be >96% pure. Prepurified nitrogen was obtained from the Matheson Company, Inc.

**Amperometric Titration of Thiols**—The titration was carried out by using a dropping mercury electrode as the indicator electrode and a saturated calomel electrode as the reference half-cell (Cecil (4) and references quoted therein). Potentials were applied and currents measured with a Fisher Electrodipode, model 65. An oxygen-free solution of the thiol in a suitable buffer was maintained at 40° in a water-jacketed polarographic cell under a slow flow of nitrogen. The cell was provided with a tightly fitting Teflon cap and a drain tap for the removal of waste mercury. The apparatus is shown in Fig. 1. The oxygen content of the nitrogen was further reduced by passage through Fieser's solution, followed by solutions of lead acetate and mercuric chloride to remove possible thiol impurities (Fieser (5)). The nitrogen was then equilibrated at 40° by passage through a solution similar to that in the cell but containing no thiol, and finally filtered through glass wool to remove spray. The gas entered and left the cell through 22-gauge Teflon tubing which fitted tightly through holes in the cap.

Mercuric chloride solution (10 mM) was used as the titrant in all cases and was added from a manually operated microburette in 1- to 5-μl aliquots. Effective stirring was obtained with a propeller made by flattening one end of a piece of heated Teflon tubing (22 gauge). The flattened end served as the stirrer and the rest of the tube, strengthened by the insertion of a length of 22-gauge copper wire, provided a flexible drive for connection to a stirring motor as shown in Fig. 1. A stirrer was added to the solution for 1 minute, followed by a rest period of 30 seconds, after which the current in the electrode circuit was measured. The potential required to give a linear relationship between current and mercuric chloride under the given conditions was determined from current-voltage curves of the solution with and without excess titrant (cf. Meites (6)). Potentials are quoted in volts relative to the saturated calomel electrode.

Whenever it was necessary to titrate cysteine at an alkaline pH, the possibility of a lower titr due to oxidation of —SH groups
was avoided as follows. Direct titration of an aliquot of the thiol gave the electrode current at the end point, $i_E$, which is essentially the current through the buffer alone. A second titration was then carried out after adding a fresh aliquot of the thiol to buffer containing about 1.1 equivalents of HgCl$_2$. In this way the titrant was always in excess and oxidation of $-SH$ groups was prevented. The true titer was calculated by extrapolation of the excess reagent line (see Fig. 2) to the value of the current at the end point, $i_E$ (7). In all titrations a complexing agent was present to avoid the phenomenon of "overbinding" of HgCl$_2$ to the mercuric mercaptides; at acid pH values 3.0 m chloride ion was used for this purpose, and at alkaline pH values this was replaced by 5 m Na$_2$S$_2$O$_3$ (Cecil (4)).

Oxygen-free Solutions of Proteins—If foaming was not a problem, oxygen-free nitrogen was passed through the protein solution for about 30 minutes. In some cases, however, the following procedure was adopted to avoid excessive frothing. The freeze-dried protein, together with a magnetic flea, was placed in tube B (see Fig. 3) and the solvent in tube A. Oxygen-free nitrogen was passed through the solvent and thence through tube B for about 30 minutes. The exit line from tube A was then lowered into the solvent and nitrogen pressure forced the solvent over into B, where it dissolved the protein, with the aid of stir-

![Fig. 1. Diagrammatic representation of the apparatus used in amperometric titration of thiols](image)

![Fig. 2. Amperometric titration of cysteine, showing the method of estimating the true end point in the titration of the thiol at alkaline pH. Titrations of 0.100 ml of a cysteine hydrochloride solution with 10 m HgCl$_2$ solution at -0.43 volt in 0.1 m borax, 0.1 m KCl, and 4 m Na$_2$S$_2$O$_3$. Conditions: total volume, 2.7 ml; temperature, 40°, pH 9.2; drop time, 3.44 seconds (open circuit).](image)

![Fig. 3. Method of preparation of oxygen-free solutions of protein.](image)
ring by the flea. The protein solution could be frozen out from D by lowering the exit line into the solution.

**Performic Acid Oxidation**—The procedure of Moore (8) was employed, and the analytical results corrected for a 94% yield of cysteic acid.

**Preparation of Samples for Amino Acid Analysis**—Salt-free samples were hydrolyzed for 22 hours (Moore and Stein (9)) and analyzed on the automatic amino acid analyzer according to the procedure of Spackman, Stein, and Moore (10). The values for tyrosine, threonine, and serine were corrected for the approximate losses of 5, 5, and 10%, respectively, during 22 hours of acid hydrolysis (9).

### RESULTS

**Estimation of —SH Groups in Acid-denatured Zymogen**—Lyophilized zymogen (14.5 mg) was dissolved in 2.6 ml of oxygen-free sodium acetate buffer (0.25 M, pH 5.6) that contained urea (8 M), KCl (2 M), and EDTA (10 mM). The protein solution was transferred under nitrogen to the polarographic cell and titration at −0.40 volt gave a titer of zero. Under these conditions, 0.2 mole of —SH groups per mole of protein would have been detected. In a further experiment, lyophilized zymogen (35.0 mg) was dissolved in 3.0 ml of sodium formate buffer (0.1 M, pH 3.0) containing guanidinium chloride (5 M). After passing oxygen-free nitrogen through the solution to remove dissolved oxygen, titration at −0.20 volt also gave a zero titer. In this case 0.1 mole of —SH groups per mole would have been detected.

**Reaction of Performic Acid with Thioester**—Before testing directly for the possible presence of a thioester group in the protein, it was important to determine whether a thioester involving the side chain of cysteine would yield cysteic acid upon oxidation by performic acid. S-Acetylglutathione was chosen as the model compound for the study of this reaction. The sample used contained 1.4% of glutathione by amperometric titration at pH 3 in the presence of performic acid. S-Acetylglutathione was chosen as the model compound for the study of this reaction. The sample used contained 1.4% of glutathione by amperometric titration at pH 3 in the presence of performic acid. S-Acetylglutathione was chosen as the model compound for the study of this reaction. The sample used contained 1.4% of glutathione by amperometric titration at pH 3 in the presence of performic acid. Therefore, 0.2 mole of —SH groups per mole of protein would have been detected. In a further experiment, lyophilized zymogen (35.0 mg) was dissolved in 3.0 ml of sodium formate buffer (0.1 M, pH 3.0) containing guanidinium chloride (5 M). After passing oxygen-free nitrogen through the solution to remove dissolved oxygen, titration at −0.20 volt also gave a zero titer. In this case 0.1 mole of —SH groups per mole would have been detected.

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**Estimation of Disulfide Groups in the Acid-denatured Zymogen**—The presence of only one half-cystinyl residue per molecule means that if a disulfide bond is present in the protein, it must be a mixed disulfide, protein—S—SR, where —SR is not a half-cystinyl residue. Were this the case, two consequences should follow. In the first place, reduction of such a disulfide by excess cysteine, followed by air oxidation of the mixture would give rise to a new mixed disulfide Cys—SR, and should introduce a second half-cystinyl residue into the protein. To examine this possibility the zymogen (10.5 mg) was dissolved in 3.0 ml of a solution containing cysteine hydrochloride (9.5 mg) and urea (1.0 g) adjusted to pH 7.22 with 1.0 N NaOH. After 20 hours at room temperature, the solution was applied to a column (2 × 40 cm)
of Sephadex G-75, medium particle size, equilibrated with 50% acetic acid. The unretarded protein fractions were pooled, rotary evaporated to dryness, and subjected to performic acid oxidation, hydrolysis, and amino acid analysis. As expected, 2.03 residues of cysteic acid per molecule were found. The retarded material from the Sephadex G-75 column was rotary evaporated to dryness, dissolved in pH 2.2 citrate buffer, and applied to the 150-cm column of the amino acid analyzer. No ninhydrin-positive peaks other than those found in a control analysis of air-oxidized cysteine were found, namely cystine and a small amount of cysteic acid. If the mixed disulfide, CySSR, was present it chromatographed in the same place as cystine.

In the second place, if a disulfide bond is present in the zymogen, it should be possible to titrate two —SH groups per molecule after reduction of the protein with sodium borohydride.

For titration, the zymogen (38.7 mg, dry weight) was placed in the polarographic cell and dissolved under nitrogen in 2.5 ml of a solution containing sodium borohydride (1% w/v), urea (8 M), KCl (2 M), EDTA (10 mM), and boric acid (78 mM), pH 9.55. The solution was left for 90 minutes at 40°C under nitrogen with the electrodes raised out of the solution. Excess sodium borohydride was decomposed by adding deoxygenated 50% acetic acid (0.1 ml) to give a final pH of 5.7. The electrodes were lowered into the solution and amperometric titration at —0.22 volt gave a titer of 0.0375 ml of 10 mM HgCl₂, equivalent to 0.89 mole of —SH groups per mole of protein (mol. wt. 44,000). A control experiment with ribonuclease under identical conditions gave a titer of 8.16 moles of —SH per mole of ribonuclease (chromatographic analysis gives 8 half-cystine residues).

During a repetition of this experiment, it was observed that the waste nitrogen leaving the cell during the first few minutes of the reduction smelled strongly of a mercaptan. It seemed possible that the second of the two expected —SH groups was present as a volatile mercaptan that was removed from the cell in the nitrogen stream. In order to estimate this mercaptan, a trap containing mercurlc chloride solution was placed in the waste nitrogen line from the cell (see Fig. 1). After reduction of the protein in the cell, the amount of mercaptan trapped as its mercuric mercaptide, (RS)₂Hg, was estimated by measuring the loss of HgCl₂ from the trap in the following way. A known amount of HgCl₂ (about 4 equivalents per mole of protein) was placed in the trap and reduction of the protein was carried out as described above. Assuming 1 equivalent of RSH to have been trapped, there would be about 3 equivalents of HgCl₂ remaining. The contents of the trap were then transferred to the polarographic cell and a solution containing about 2.8 equivalents of cysteine was added (i.e. a known volume of a cysteine solution freshly standardized by titration with HgCl₂). This was done in order to reduce the excess of HgCl₂, but care was taken to ensure that HgCl₂ always remained in excess; otherwise the volatile RSH might have been released and lost. The small excess of HgCl₂, x equivalents, now remaining (x is about 0.2 equivalent) was determined amperometrically by titrating the solution with aliquots of standard HgCl₂ solution and extrapolating the resulting straight line back to the known current, i₀, in the absence of HgCl₂, as shown in Fig. 4.

A series of experiments was carried out in the above manner, with 15 to 25 mg of protein in each case. The excess sodium borohydride was decomposed by adding deoxygenated acetone (0.2 ml) followed after 20 minutes by deoxygenated 50% acetic acid (0.1 ml) to lower the pH to 5.7. The use of acetone avoids the excessive evolution of hydrogen and concomitant frothing that was experienced when acetic acid was used to decompose the borohydride. In all other respects these experiments were similar to the one described above. The results are given in Table I. The low titer for the volatile mercaptan are probably a result of inefficiency of the trapping system used.

In order to estimate the volatile mercaptan more accurately a closed system was employed. Sodium borohydride could not be employed as the reducing agent under these conditions because of the release of hydrogen from the reagent. Cysteine or sodium sulfite were therefore used in its place.

The zymogen (30 to 40 mg) was dissolved under nitrogen in 3.0 ml of a solution containing urea (8 M), Na₂HPO₄ (50 mM), and NaH₂PO₄ (50 mM), and transferred under nitrogen to a Warburg flask. In the center well of the flask, 0.15 ml of HgCl₂ solution (10 mM) were placed, together with 0.2 ml of 0.1 M borax, 0.1 ml KCl, and 0.1 ml of 0.1 M Na₂S₂O₃. The side arm of the flask contained 30 mg of cysteine hydrochloride. When sodium sulfite was used as the reducing agent the protein was dis-
Volatile mercaptan formed on reduction of zymogen

The volatile mercaptan, RSH, was trapped in HgCl₂ solution in the well of a closed Warburg flask containing acid-denatured zymogen and a reducing agent under nitrogen. The values were estimated by amperometric titration of the HgCl₂ remaining in the well, as described in the text. A correction has been applied for the loss of HgCl₂ from the well in control experiments without protein.

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>pH</th>
<th>Temperature</th>
<th>Duration</th>
<th>RSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>7</td>
<td>25 °C</td>
<td>hrs</td>
<td>24</td>
</tr>
<tr>
<td>Cysteine</td>
<td>9</td>
<td>25 °C</td>
<td>72</td>
<td>1.27</td>
</tr>
<tr>
<td>Na₂SO₃</td>
<td>9</td>
<td>40 °C</td>
<td>72</td>
<td>1.09</td>
</tr>
</tbody>
</table>

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DISCUSSION

The volatile nature of the mercaptan, RSH, renders its detection and estimation difficult when one considers that 44 mg of the zymogen will liberate only 1 μmole of RSH. For example, its volatility explains why after reducing the mixed disulfide, protein-S-SR, with excess cysteine followed by air oxidation, no new mixed disulfide, CyS-SR, was found in the solution. Although the mercaptan is volatile, it cannot be very low boiling, since 72 hours are required for it to distill completely in the closed system (Table II). With a stream of nitrogen passing through the solution, as in the reductions in the titration cell, the mercaptan is carried over more rapidly.

An explanation can be provided for the observed release of a full mole of RSH per mole of protein by the action of sulfite ion in 8 M urea. The sulfitolysis of the mixed disulfide would be expected to set up two equilibria.

RS-SO₃⁻ + protein-S⁻ ⇌ protein-SR + SO₃²⁻

The irreversible removal of RSH from the mixture by distillation into the HgCl₂ trap would draw the equilibria to the right and, in the relatively long period allowed for the reaction, would yield only the right hand products.

The elusive nature of this unusual partner in a protein disulfide bond suggests that it or other volatile mercaptans might be present in other proteins. In this respect it is of interest that King (13) has found mixed disulfides involving serum albumin and cysteine or glutathione, and Huisman and Dozy (14), and Taylor, Antonini, and Wyman (15) have prepared mixed disulfides of hemoglobin with glutathione and cysteine. Westhead, Butler, and Boyer (16) found that reduction of aldolase caused the appearance of a single new —SH group, which might have been involved in a mixed disulfide of the kind found in this work.

The problem of distinguishing between mixed disulfides of this type, sterically inaccessible —SH groups, and thioesters or other compounds such as sulfenic or sulfonic acids is not always straightforward. All such compounds will be oxidized to a sulfonic acid by performic acid and all of them could give rise to a free —SH group after treatment with a large excess of a thiol as, mercaptoethanol. In general, however, sterically inaccessible —SH groups are freed by 8 M urea or even more effectively by guanidine chloride, while thioesters are converted to the free thiol by hydroxylamine, ammonia, or other bases. Disulfides are unaffected by such treatment, which provides the basis for a means of distinguishing among these three possibilities. There are however, no established methods for detecting sulfenic or sulfonic acid groups in proteins.

The question of the identity of the volatile mercaptan and the mechanism by which it becomes combined with the protein in the mixed disulfide, must await further studies. The source of the mercaptan is the culture of hemolytic streptococci which yields to the medium the extracellular proteinase, in the form of its zymogen. Mercaptans are known to participate in bacterial metabolism, and it is understandable that a volatile member of the series could be available for incorporation into the molecule of this protein.

SUMMARY

Both streptococcal proteinase and its zymogen have one residue of half-cystine per molecule. The zymogen contains no detectable —SH or thioester groups. Reduction of the zymogen by sodium borohydride or cysteine gives rise to a single protein-bound —SH group per molecule, and also one molecule of a volatile mercaptan which can be trapped in aqueous mercuric chloride. This result is taken to mean that the zymogen and the unreduced enzyme, derived from it by proteolysis, contain a mixed disulfide, protein-S-SR where RSH is volatile.

Acknowledgment—The samples of the zymogen used in these experiments were prepared by Dr. Stuart Elliott. We wish to acknowledge the generous cooperation of Dr. Elliott and Dr. Teh-Yung Lin in the course of the present study, and the technical assistance of Miss Susan Donner. We would also like to thank Mr. S. Theodore Bella who performed the elementary microanalyses recorded in this paper.

REFERENCES
9. MOORE, S., and STEIN, W. H., in S. P. COLOWICK and N. O.


