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On the Mechanism of Action of Streptococcal Proteinase. I. Active-Site Titration†

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ABSTRACT: The amount of active enzyme in proteinase solution has been determined by a number of methods including titration of the SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) and stoichiometric inhibition of the enzyme with $HgCl_2$ or iodoacetic acid. The results of these experiments are consistent with the conclusion that the SH titer of the proteinase is a direct measure of the amount of active enzyme present. Proteinase preparations from different batches of crystalline zymogen exhibited different specific activities. The sole demonstrable difference was in their thiol content. Successful separation of active mercaptoproteinase and inactive nonmercaptoproteinase has been achieved by use of a Sepharose–mercurial column originally developed by Sluyterman and Wijdenes (Sluyterman, L. A. E., and Wijdenes, J. (1970), *Biochim. Biophys. Acta 200*, 593) for the purification of active papain. The active proteinase contained one titrable SH per mole of enzyme and exhibited a maximum specific activity, while the inactive proteinase contained less than 0.05 mol of SH/mol of enzyme and failed to exhibit any enzymic activity. These results are in agreement with the suggestion that the single SH group in the proteinase is essential for its enzymic activity. Sulfite ion was found to inhibit reduced proteinase at pH 5.0 at 25°. There was a binding of nearly 1 mol/mol of SO_3^- to the protein. The mechanism of this inhibition by sulfite ion is not yet known.

It has been shown (Elliott, 1945; Elliott and Dole, 1947) that group A streptococci elaborate an extracellular zymogen which can be transformed into an active proteinase by proteolysis followed by reduction. Both the zymogen (mol wt 44,000) and the enzyme (mol wt 32,000) contain only a single "potential half-cystine" residue per molecule (Liu *et al.*, 1963). The inactive enzyme formed by proteolysis of the zymogen requires activation by exposure to reducing agents. The zymogen isolated from the streptococcal culture is homogeneous by chromatography on an ion-exchange column, N-terminal residue analysis, amino acid analysis, and ultracentrifugal analysis (Liu *et al.*, 1963). Homogeneous samples of enzyme can be obtained in high yield upon treatment of the zymogen with trypsin, which removes about 100 amino acid residues (Liu and Elliott, 1965). The SH group of the potential half-cystine residue is liberated only when the enzyme is activated by reduction. This reductive step causes no detectable change in molecular weight.

The presence of one half-cystine residue in both the zymogen and the unreduced enzyme is well established (Liu and Elliott, 1965). Performic acid oxidation followed by acid hydrolysis of the zymogen or the unreduced enzyme after activation with thiols yields one residue of cysteic acid per mole. However, it has been known for some time that essentially homogeneous zymogen or unreduced enzyme after activation with thiols yields enzymes with different specific activities. When chromatographed on SE-Sephadex C-25 such activated proteinase showed heterogeneity (Liu and Elliott, 1965), but separation of active and inactive proteinase has not been achieved.

This article is concerned with experiments designed to demonstrate that the specific activity of the activated enzyme (proteinase) is directly proportional to its SH content. Separation of active mercaptoproteinase and inactive nonmercaptoproteinase was accomplished by use of the Sepharose–mercurial column of Sluyterman and Wijdenes (1970). A preparation was obtained which contains one titrable SH group per mole of proteinase. This preparation exhibited the maximum enzymic activity obtainable.

Experimental Section

Materials. Streptococcal zymogen was prepared by Dr. S. D. Elliott in the form of an 0.8-saturated $(NH_4)SO_4$ protein precipitate of the culture medium (Elliott, 1950). The papain preparation used in a control experiment was obtained from Worthington Biochemicals, crystalline suspension, lot PAP 9DA. The preparation showed 55% of the maximum activity obtainable when assayed with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester (Z-Gly-Nph).[1] Trypsin, twice crystallized, obtained from Worthington Biochemicals,

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[1] The abbreviations used are: Z, benzyloxycarbonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; Nph, *p*-nitrophenyl ester; Ph, phenyl ester.

was treated with α-*N*-tosyl-L-phenylalanyl chloromethyl ketone (Schoellman and Shaw, 1962) to remove chymotryptic enzyme (Kostka and Carpenter, 1964).

Z-Gly-Nph was synthesized by the dicyclohexylcarbodiimide method (Bodanszky and duVigneaud, 1962). The ester was crystallized from chloroform–hexane, mp 127°. The purity of the ester was 99 ± 1% based on the amount of *p*-nitrophenol released.

Dithioerythritol was obtained from Calbiochem. Sodium tetrathionate was prepared as described earlier (Gilman *et al.*, 1946; Inglis and Liu, 1970). 5,5′-Dithiobis(2-nitrobenzoic acid), *p*-aminophenylmercuric acetate, and spectrophotometric grade dimethyl sulfoxide were purchased from Aldrich Chemical Co. Iodoacetate was obtained from K&K Laboratories.

[^{35}S]Sulfite, 10–50 mCi/mmol, purchased from New England Nuclear, was diluted with unlabeled compound from Eastman to a specific activity of 1 mCi/mmol. [^{14}C]Phenylhydrazine HCl, 5–10 mCi/mmol, from Tracerlab, Richmond, Calif., was diluted with unlabeled compound from Eastman to a specific activity of 500–800 cpm/mμmol and recrystallized from ethanol. Hydroxylamine HCl was purchased from Fisher Scientific Co. and recrystallized from ethanol.

Methods. Protein concentrations were calculated from absorbance measurements using an extinction coefficient of $A^{1\%}_{280,1\text{ cm}} = 16.4$ for streptococcal proteinase (Liu *et al.*, 1963), $A^{1\%}_{280,1\text{ cm}} = 13.7$ for streptococcal zymogen (Liu *et al.*, 1963), and $A^{1\%}_{278,1\text{ cm}} = 25.0$ for papain (Glazer and Smith, 1961). Enzymic activities were measured routinely with Z-Gly-Nph as the assay substrate. To 3.0 ml of sodium acetate buffer, pH 5.50, I = 0.2, equilibrated at 25 ± 0.1° in a cuvet in the sample compartment of the Cary-15 spectrophotometer, was added 0.1 ml of Z-Gly-Nph in acetonitrile (2.78×10^{-3} M), and the mixture was stirred with a glass rod. Enzyme solution, 50 μl (5–20 μg), on a glass rod was added to initiate the reaction. The hydrolysis of Z-Gly-Nph was measured by observing the release of *p*-nitrophenol at 317 mμ. Under these conditions no significant spontaneous hydrolysis of the substrate takes place during the course of the reaction. One unit of activity for proteinase is defined as the amount of enzyme required to cause the change in absorbance, $A_{317,\,1\text{ cm}}$ per minute per milliliter of enzyme, using Z-Gly-Nph as substrate at 25° and pH 5.5 when $[S]_0 = 7.58 \times 10^{-5}$ M. The specific activity is defined as activity in units per milliliter per $[E]_0$ in milligrams/milliliter. In several experiments, α-*N*-Z-L-Lys-Ph ($[S]_0 = 1.12 \times 10^{-3}$ M) was also used as an assay substrate. The assay conditions were the same as for Z-Gly-Nph and the release of phenol was followed at 270 mμ. The activity ($\Delta A_{270,\,1\text{ cm}}$ per minute per milliliter) and specific activity are as defined above.

Radioactivity measurements were made with a Packard Tri-Carb scintillation counter. For bottle counting, 0.2-ml samples were counted in 10 ml of a solution containing 5 g of 2,5-diphenyloxazole and 2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene–ethanol mixture (Hall and Cocking, 1965). The amount of radioactive compound bound to the protein was estimated from a standard curve constructed with values obtained from counting known amounts of radioactive chemicals.

Purification of the Zymogen. The half-saturated $(NH_4)_2SO_4$ precipitate of the zymogen was thawed by allowing it to stand at 37° for 24 hr and centrifuged to yield the 0.5 zymogen fraction. The supernatant was brought to 0.8 saturation in $(NH_4)_2SO_4$ and the resultant protein precipitate was collected by centrifugation to yield the 0.5–0.8 zymogen fraction. These zymogen precipitates were dissolved in a minimum volume of 0.04 M NaCl–0.001 M EDTA containing 1 mg/ml of sodium tetrathionate and subjected to gel filtration on a Sephadex G-100 column (85 × 2.5 cm) equilibrated with 0.04 M NaCl–0.001 M EDTA (Figure 1).

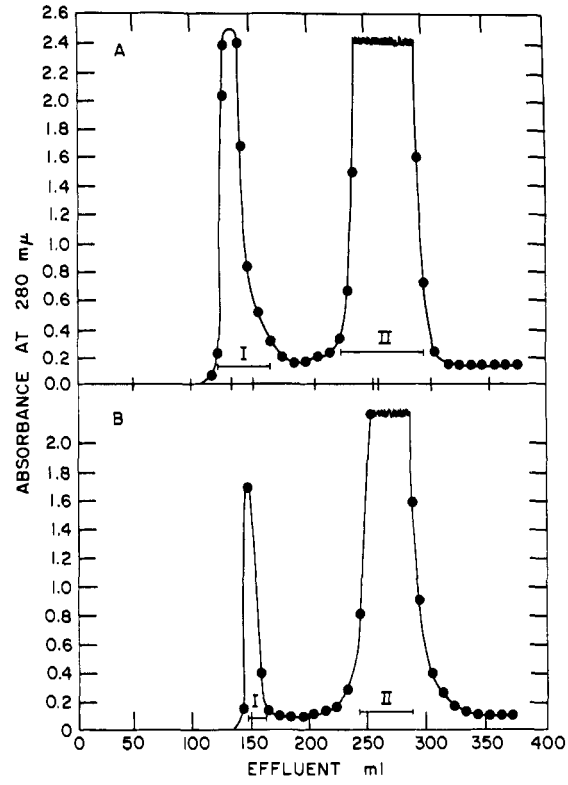

FIGURE 1: Gel filtration of the zymogen of streptococcal proteinase on columns of Sephadex G-100 (2.5 × 85 cm) in 0.04 M NaCl–0.001 M EDTA: (A) 0.5-saturated $(NH_4)_2SO_4$ fraction; (B) 0.5–0.8-saturated $(NH_4)_2SO_4$ fraction.

Conversion of the Zymogen to Enzyme. The conversion of zymogen to enzyme by the proteolytic action of trypsin at pH 6.7 ± 0.1 has been described by Liu and Elliott (1965). This method at times results in incomplete conversion partly because of the presence of the aggregated form of the zymogen and also because of the nonoptimum condition used for trypsin. Although proteinase can be readily separated from the zymogen and its modified products on SE-Sephadex C-25, the yields of enzyme are usually lower. The present studies indicate that a much higher yield can be obtained when the conversion is carried out at pH 8.5 rather than 6.7. The resulting unreduced enzyme is homogeneous by SE-Sephadex C-25 and electrophoresis on cellulose acetate (Liu and Elliott, 1965). To obtain active enzyme, this enzyme still requires reduction with a thiol compound such as DTE.

Transformation by Autodigestion. To a solution of zymogen (1–2 mg/ml) in 0.04 M NaCl was added 0.1 vol of 1 M phosphate buffer, pH 6.7, containing 0.01 M EDTA and 1.5 mg/ml of DTE, and the resulting solution was incubated at 37°. Under these conditions maximum enzymic activity was attained in 70 min (Figure 2). At maximum activity, sodium tetrathionate, 3.0 mg/ml, was added to convert the reduced, active form of the enzyme to the inactive *S*-sulfenyl sulfonate derivative (Liu, 1967). The solution was dialyzed against 3 × 4 l. of 0.04 M NaCl–0.01 M EDTA to remove excess reagents and low molecular weight peptides produced by

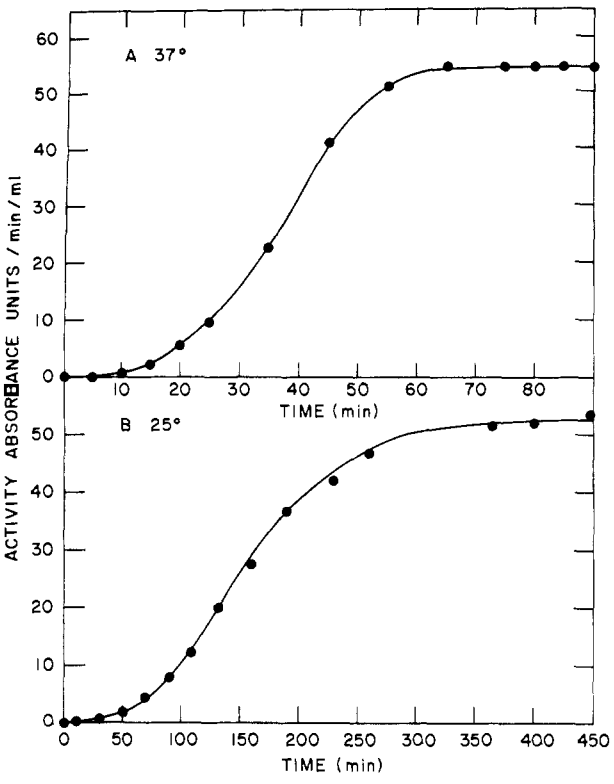

FIGURE 2: Activation of zymogen by autocatalysis. (A) Reaction carried out at 37°, 13.2 mg of zymogen in 7.0 ml of 0.1 M phosphate buffer, pH 6.7 containing 0.001 M DTE. Appearance of enzymic activity was followed by assaying 0.025-ml aliquots of the reaction mixture at given time intervals using α-*N*-Z-Lys-Ph, $[S]_0 = 1.23 \times 10^{-3}$ M, as substrate. Activities are expressed as ΔA per minute per milliliter of enzyme. (B) Reaction carried out at 25°.

autolysis. The *S*-sulfenylsulfoproteinase was further purified by passage through Sephadex G-100 and SE-Sephadex columns. When kept at −20°, this form of proteinase is stable indefinitely and can be reactivated by exposure to 0.001 M DTE at pH 5.5 and 25° for 60 sec.

To obtain activator-free proteinase, the reduced enzyme is dialyzed against oxygen-free 0.04 M NaCl–0.001 M EDTA solution at 4°. Oxygen-free solution is prepared by boiling out oxygen and saturating the solution with oxygen-free nitrogen. When stored under nitrogen, the activator-free reduced proteinase is stable for several weeks at 4°.

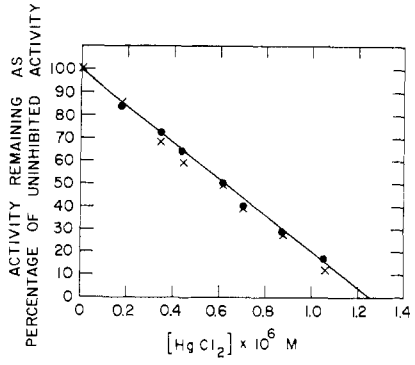

FIGURE 3: Inhibition of reduced proteinase with $HgCl_2$ at pH 4.73 (×) and pH 6.2 (●). $[E]_0 = 1.275 \times 10^{-6}$ N (by DTNB titration). Extrapolation gives complete inhibition at 1.245×10^{-6} M $HgCl_2$. The ratio $[HgCl_2]$/[SH groups] is 0.976.

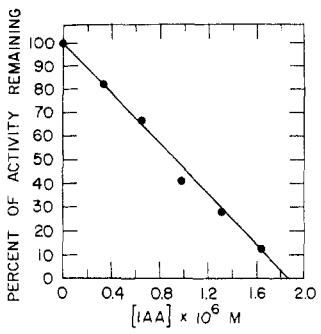

FIGURE 4: Inhibition of reduced proteinase with iodoacetic acid in phosphate buffer, pH 6.205, $I = 0.2$ at 25°. Aliquots of 50 μl were withdrawn for assay against Z-Gly-NPh, $[E]_0 = 2.00 \times 10^{-6}$ N (determined by DTNB titration). Extrapolation gives complete inhibition at [IAA] of 1.86×10^{-6} M. The ratio [IAA]/[SH groups] is 1.08.

Titration of Active-Site Thiols with DTNB and $HgCl_2$. The concentration of thiol groups in the reduced enzyme was determined by the method of Ellman (1959). The reaction mixture, which was placed in a cuvet, contained 3×10^{-3} μmol of proteinase in 3.0 ml of 0.05 M Tris-HCl buffer, pH 8.0, and 0.025 ml of 0.01 M DTNB in 0.05 M phosphate buffer, pH 7.0. The reaction was carried out at 25°, and the release of 2-nitro-5-mercaptobenzoic acid was followed at 412 mμ, using the 0–0.1 absorbance slide-wire of the Cary-15 spectrophotometer. A value of 13,600 was used for the molar absorptivity of 2-nitro-5-mercaptobenzoic acid. The titration of thiols was also done in 8 M urea or 0.2% sodium dodecyl sulfate in 0.05 M Tris-HCl buffer. The molarity of the proteinase was calculated by assuming a molecular weight of 32,000 and $A^{1\%}_{280,\ 1\ cm} = 16.4$ (Liu *et al.*, 1963).

For experiments with $HgCl_2$, the reduced enzyme is dialyzed against oxygen-free 0.04 M NaCl at 4° to remove the EDTA. The stoichiometry of inhibition of proteinase by $HgCl_2$ was studied at pH 4.73 and 6.21 (Figure 3). To a series of reaction tubes containing 7×10^{-4} μmol of reduced proteinase in 0.53 ml of buffer, varying volumes of $HgCl_2$ (1.046×10^{-5} M) were added to a final volume of 0.6 ml. After mixing, the residual enzymic activity was assayed in duplicate with 0.05-ml aliquots of the reaction mixture.

Inhibition with Iodoacetate. A similar experiment was set up to determine the stoichiometry of the inhibition of reduced proteinase with iodoacetic acid in 0.1 M phosphate buffer, pH 6.21 (Figure 4). Treatment of the reduced proteinase with an equimolar amount of iodoacetic acid resulted in complete loss of enzymic activity. The inactivated proteinase was desalted on a Sephadex G-25 column and analyzed for *S*-carboxymethylcysteine content after acid hydrolysis (Crestfield *et al.*, 1963).

Sepharose–Mercurial Column Procedures. The method of Cuatrecasas *et al.* (1968) as modified by Sluyterman and Wijdenes (1970) was used for the preparation of Sepharose–mercurial. To 100 g of Sepharose (Sepharose 4B, Pharmacia, Sweden) mixed with an equal volume of water was added cyanogen bromide (100 mg/ml of settled Sepharose) in an equal volume of water. The pH was immediately adjusted to, and maintained at, 11 by titration with 4 M NaOH. When the reaction ended (about 10 min), the Sepharose was washed with 20 vol of cold 0.1 M $NaHCO_3$ on a Büchner funnel under suction. The washed Sepharose was suspended in 200 ml of 10% dimethyl sulfoxide at 0°, and 1.3 g of *p*-

aminophenylmercuric acetate in 20 ml of dimethyl sulfoxide was added. The suspension was stirred slowly and stored at 4° for 20 hr, warmed up to 30°, and suction filtered. The Sepharose was resuspended in 130 ml of 20% dimethyl sulfoxide at 35° for 5 min and filtered off. This treatment was repeated four times.

Each column of Sepharose–mercurial to be used for the purification of activated proteinase or papain was packed (0.9 × 60 cm) and washed with 20% dimethyl sulfoxide until no more mercurial appeared in the effluent. The column was then washed with phosphate buffer (pH 8.0, $I = 0.1$) followed by a solution of 2.5 mM DTNB in the pH 8.0 phosphate buffer until the eluate had the same yellow color as the initial solution. The column was next washed with the phosphate buffer until the eluate was colorless and equilibrated with 0.05 M sodium acetate buffer of pH 5.0. A solution of 2 mM $HgCl_2$ in the pH 5.0 acetate buffer was passed through the faintly yellow column until the column became colorless. To estimate the capacity of the column, an aliquot of the effluent was mixed with a solution of 0.1 M cysteine at pH 8.5 and the absorbancy at 412 mμ was read. This treatment prevents the irreversible binding of SH protein to the column. A Sepharose–mercurial column prepared in this manner has a capacity of 1.5 mmol/ml for the thiol reagent. The column can be regenerated by recycling with $HgCl_2$ and sodium acetate buffer for repeated use.

In a typical experiment, the streptococcal zymogen or a crystalline suspension of papain was activated with a solution of DTE at 37° for 60 min as described above. The reagents were removed by dialysis against 0.04 M NaCl in 0.001 M EDTA under N_2, and the enzyme was added to the column. The column was eluted with the pH 5.0 sodium acetate buffer (10% dimethyl sulfoxide, 0.5% *n*-butyl alcohol, 0.1 M KCl, and 0.05 M sodium acetate) containing 0.001 M EDTA until the absorbancies at 280 mμ of the effluent and the initial buffer were equal. Then the active enzyme bound to the column was eluted with the pH 5.0 sodium acetate buffer containing 0.5 mM $HgCl_2$ instead of 0.001 M EDTA (Figure 5).

Reaction of Reduced Proteinase with Na_2SO_3. In the procedure of Sluyterman and Wijdenes (1970) for the purification of papain on a Sepharose–mercurial column, activator-free papain was prepared in 0.05 M sodium acetate buffer, pH 5.0, containing 0.001 M EDTA and 10 mM Na_2SO_3. When reduced activator-free streptococcal proteinase or papain was equilibrated with this buffer in a dialysis sac under N_2 at 25° for 18 hr, enzymic activity against Z-Gly-Nph was completely lost. Reincubation of the inactivated proteinase or papain with DTE (1×10^{-3} M) at 25° for 2 hr at pH 5.5 in sodium acetate buffer did not result in regeneration of enzymic activity. It was found, however, that if Na_2SO_3 was eliminated from the buffer, enzymic activity was retained. Experiments were therefore designed to examine the effect of Na_2SO_3 on the activity of reduced proteinase.

Streptococcal proteinase recovered from the Sepharose–mercurial column was activated with DTE and prepared free of Hg^{2+} dialyzing against 0.04 M NaCl in 1 mM EDTA. Reduced proteinase (3 mg/ml) was then equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 0.001 M EDTA and 10 mM [^{35}S]sulfite under N_2 for 18 hr at 25°. At the end of this period, enzymic activity was examined with Z-Gly-Nph as a substrate. The [^{35}S]sulfite-treated reduced proteinase was then eluted through a Sephadex G-25 column (0.9 × 85 cm) with 0.04 M NaCl and 1 mM EDTA (Figure 6). Portions of the [^{35}S]sulfite-treated proteinase were treated with 0.01 M DTE at pH 5.5 in 0.05 M sodium acetate buffer for 2 hr at 25° prior to chromatography on a Sephadex G-25 column. The effluent was examined for both radioactivity and protein concentration.

Results

Properties of Zymogen. Sephadex G-100 gel filtration patterns of the 0.5 and 0.5–0.8 zymogen fractions are shown in Figure 1. For both preparations, two protein peaks were observed. Peak II, containing 80–90% of the applied protein, was eluted in about 60% of the column volume. In a calibration experiment, ovalbumin (mol wt 45,000) was eluted in 57% of the column volume. The protein in peak II was, therefore, identified as the zymogen (mol wt 44,000) (Liu *et al.*, 1963). The proteins in peak II from both the 0.5 and the 0.5–0.8 zymogen fractions showed the same amino acid composition. Peak I, containing 10–20% of the applied protein, was eluted in the void volume of the column. The amino acid composition of peak I was identical with that found for the zymogen in peak II. Upon treatment of the protein in peak I with 12 M urea at pH 6.7 in 0.1 M phosphate buffer at 25° for 6 hr and rechromatography of the urea-treated protein on Sephadex G-100, 65–70% of the protein was recovered in the peak II position. Thus, the protein in peak I is probably an aggregate form of the zymogen, with an apparent molecular weight higher than 100,000. Since the aggregate can be dissociated into monomer with urea, protein dimer involving disulfide linkage of the type zymogen–S–S–zymogen is unlikely to be present in peak I.

The aggregated form of the zymogen has not been found in every batch of zymogen preparation, and its amount varies from one preparation to another. That it plays a significant role in the metabolism of group A streptococcus is unlikely.

Properties of Active Proteinase. Active proteinase can be prepared either by autodigestion of the zymogen in the presence of thiol or by reduction of the unreduced enzyme obtained from the tryptic digestion of zymogen (Liu and Elliott, 1965). The amino acid compositions of the active enzyme produced by the two different procedures are almost identical. A time course of the process involving the autocatalytic activation of zymogen is shown in Figure 2. Maximum enzymic activity was reached in 70 min at 37° and in 400 min at 25° under the experimental conditions used.

Proteinase activity can be rapidly assayed by measuring the esterase activity. The substrate of choice is Z-Gly-Nph. Other substrates such as *N*-Z-Ala-Nph or α-*N*-Z-Lys-Ph have also been used in the assay of proteinase (Liu *et al.*, 1969; Kortt and Liu, 1973a,b). Z-Gly-Nph was preferred in the present study since it is readily available in high purity and reasonably soluble in water, and exhibits suitable kinetic constants for routine work.

Proteinase preparations from different batches of crystalline zymogen were found to exhibit specific activities from *ca.* 3.0 to 7.2 upon activation. Activity was not lost upon dialysis to remove the DTE. The reduced enzyme, when stored under N_2, lost less than 10% of its activity over a period of 4 weeks. Titration of enzyme solutions of different specific activities showed that the concentration of thiols, as measured by the release of 2-nitro-5-thiobenzoic acid followed at 412 mμ, was directly proportional to the concentration of active enzyme. Table I lists the specific activities and SH contents of various enzyme preparations, and shows that the activity is proportional to the sulfhydryl group content. Titration of sulfhydryl groups in the enzyme therefore gives a measure of the normality of the active enzyme, and the molar ratio

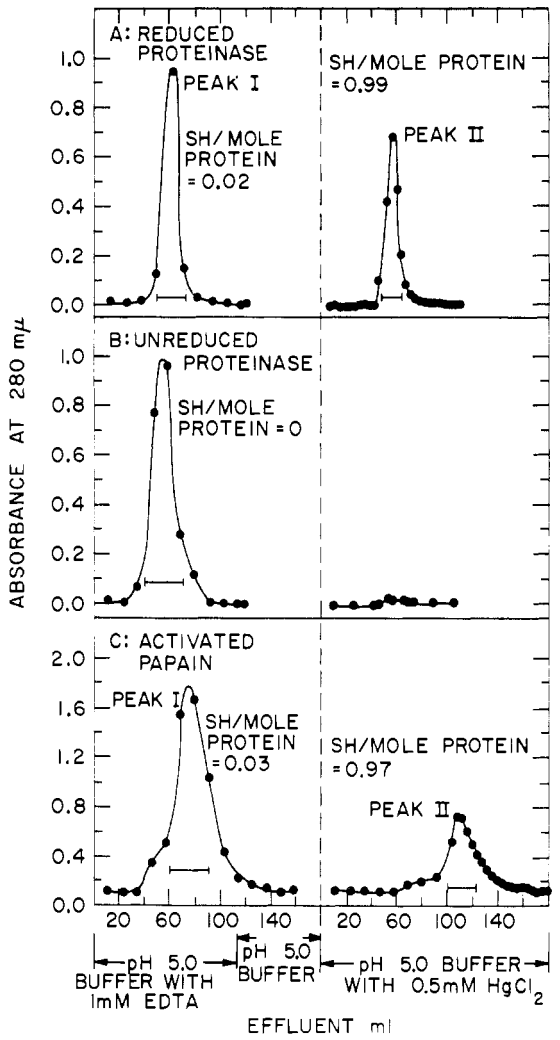

FIGURE 5: Separation of active mercaptoproteinase from inactive nonmercaptoproteinase on columns of Sepharose–mercurial (0.9 × 50 cm). See text for experimental details: (A) activated streptococcal proteinase; (B) unreduced streptococcal proteinase; (C) activated papain solution from Worthington Biochemicals.

of SH to protein enzyme gives the fraction of active enzyme in a particular preparation. From the normality, molarity, and specific activity of an enzyme solution, the maximum specific activity of proteinase with one SH group per mole of enzyme can be calculated. This value was 10.5 ± 0.5 for proteinase when Z-Gly-Nph was used as the substrate and 105 ± 5 when α-*N*-Z-Lys-Ph was used as the substrate (Table I).

In the titration of thiols with DTNB, the same values were obtained in the presence or absence of either 8 M urea or 0.2% sodium dodecyl sulfate. However, the rate of reaction of the SH groups with DTNB was similar in the presence or absence of 8 M urea but was markedly decreased in the presence of 0.2% sodium dodecyl sulfate. This agrees with the previous finding that the enzymic activity of the proteinase is not destroyed in 8 M urea but is destroyed in 0.2% sodium dodecyl sulfate (Liu and Elliott, 1971). The unusual reactivity of the thiol in the proteinase toward iodoacetic acid was maintained in 8 M urea but not in 0.2% sodium dodecyl sulfate.

Stoichiometric Inhibition of Proteinase with $HgCl_2$ and Iodoacetate. The results of the stoichiometric inhibition of proteinase by $HgCl_2$ at pH 4.73 and 6.21 are shown in Figure 3. The active enzyme concentration used in this experiment was 1.275×10^{-6} N as determined by DTNB titration. The data in Figure 3 reveal that complete inhibition of the proteinase was achieved at 1.245×10^{-6} M $HgCl_2$ which gave the ratio [$HgCl_2$]/[SH groups] = 0.976. A similar experiment was set up to determine the stoichiometry of the inhibition of reduced proteinase with iodoacetic acid. The result obtained as shown in Figure 5 revealed that complete inhibition was achieved at an iodoacetic acid concentration of 1.86×10^{-6} M. The normality of the enzyme used in this experiment was $[E]_0 = 2.00 \times 10^{-6}$ N as determined by DTNB titration. This gives the ratio [IAA]/[SH groups] = 1.08. Amino acid analyses of the iodoacetate inhibited enzyme showed that the yield of *S*-carboxymethylcysteine corresponds to the amount of SH groups present in the proteinase. Earlier, it had been shown (Liu *et al.*, 1965; Gerwin, 1967) that inactivation of streptococcal proteinase by iodoacetate resulted solely in the formation of *S*-carboxymethylcysteine.

Separation of Mercapto- and Nonmercaptoproteinase. When the activated proteinase and papain were subjected to chromatography on the Sepharose–mercurial column, the elution pattern exhibited two major peaks (Figure 5). Assay for the esterase activity showed that peak I for both proteinase and papain was negligible. Titration of peak I with DTNB indicated less than 0.05 mol of SH/mol of protein. The enzymes in peak II for proteinase and papain exhibited maximum activity. Titration of SH groups in peak II proteins revealed a SH/protein ratio of 0.99 for the proteinase and 0.97 for papain. Figure 5B shows the elution profile of a sample of unreduced enzyme. As expected, the unreduced proteinase with the SH group masked (Ferdinand

TABLE I: Specific Activities and Sulfhydryl Group Concentration of Reduced Proteinases.[a]

Enzyme Preparations	Specific Activity	SH Group[d]/Mol of Proteinase	Calculated Specific[e] Activity for Enzyme with 1 SH Group/Mol
E-78	59.1[b]	0.56	106
E-82	52.6[b]	0.49	107
	57.2[b]	0.51	112
	40.3[b]	0.37	106
E-82	3.93[c]	0.37	10.4
E-85A	5.89[c]	0.56	10.5
E-93	4.65[c]	0.44	10.6
E-95	5.12[c]	0.48	10.7
	5.01[c]	0.48	10.5
	4.62[c]	0.42	10.9
	4.86[c]	0.44	11.1

[a] See text for the preparation of reduced proteinases. [b] Activities determined by using α-*N*-Z-Lys-Ph as substrate, $[S]_0 = 1.12 \times 10^{-3}$ M. [c] Activities determined by using *N*-Z-Gly-Nph as substrate, $[S]_0 = 7.56 \times 10^{-5}$ M. [d] Determined by titration with DTNB and assuming a mol wt of 32,000 for the proteinase as described in the text. [e] Values calculated from specific activities and SH group/mol of proteinase determined experimentally.

TABLE II: Amino Acid Composition of Streptococcal Proteinase.[a]

Amino Acid	Residues per Molecule: Unreduced Proteinase[b]	Reduced Proteinase[b] Peak I	Reduced Proteinase[b] Peak II
Aspartic acid	38.7	39.6	41.3
Threonine	10.6	10.4	9.87
Serine	23.0	23.1	24.4
Glutamic acid	28.5	28.0	29.5
Proline	15.6	14.9	15.1
Glycine	36.5	37.7	36.7
Alanine	21.8	22.2	22.3
Half-cystine	1.02[c]	0.38,[c] 0.45[d]	1.15,[c] 1.08[d]
Valine	21.9	21.2	22.8
Methionine	4.66, 4.95[c]	4.51, 4.87[c]	4.59, 4.86[c]
Isoleucine	12.8	12.3	13.2
Leucine	17.0	17.0	17.0
Tyrosine	17.9	18.1	18.2
Phenylalanine	11.7	11.8	12.2
Tryptophan	4.0	4.01	4.1
Lysine	17.0	17.0	17.0
Histidine	7.71	7.67	7.8
Arginine	8.6	8.55	8.2

[a] Determined by ion-exchange chromatography (Spackman *et al.*, 1958). Proteins were hydrolyzed with 4 N methanesulfonic acid at 115° for 22 hr (Liu and Chang, 1971; Liu, 1972). Average of duplicate analyses. The results are expressed as the calculated number of residues per molecule, based on lysine equals 17.0 for the short column and leucine equals 17.0 for the long column. [b] See Figure 5 for the proteinase sample used in the analyses. [c] Measured as cysteic acid or methionine sulfone after performic acid oxidation (Moore, 1963). [d] Measured as *S*-sulfocysteine (Inglis and Liu, 1970).

et al., 1965) was eluted in the position of the inactive nonmercaptoproteinase. These results are in agreement with the report of Sluyterman and Wijdenes (1970), who first demonstrated the effectiveness of the Sepharose–mercurial column for the separation of mercapto- and nonmercaptopapain. The procedure is reproducible. Portions of the proteinase solution recovered from both peaks I and II were desalted on a 40 × 2 cm column of Sephadex G-25 in 5% acetic acid. The salt-free protein solutions were evaporated to dryness, and the residues were subjected to amino acid analysis before and after performic acid oxidation. The results are shown in Table II.

Binding of [^{35}S]Sulfite to Reduced Proteinase. The extent of binding of [^{35}S]sulfite to reduced proteinase is shown in Table III. There is almost 1 mol/mol of [^{35}S]sulfite bound to the fully active proteinase (peak II proteinase). The binding of [^{35}S]sulfite to the reduced proteinase is not reversed by treatment with DTE. This derivative was enzymatically inactive when assayed against Z-Gly-Nph. The results shown in Table III suggest that [^{35}S]sulfite is bound to both the active and inactive proteinase although the binding to the inactive proteinase is less than 0.2 mol/mol of protein.

Chromatography of [^{35}S]sulfite-treated proteinase is shown in Figure 6. The elution profiles show that the protein-bound [^{35}S]sulfite is well separated from the nonprotein-bound [^{35}S]sulfite and that the binding of [^{35}S]sulfite to the active proteinase is most unlikely due to artifact.

TABLE III: Reaction of Proteinase with [^{35}S]Sulfite.[a]

Enzyme Preparations[b]	[^{35}S]Sulfite Bound[c] per Mol of Reduced Proteinase: Before Treatment with DTE	After Treatment with DTE
Peak I from	0.15	0.13
Sepharose–mercurial column	0.17	0.15
Peak II from	1.03	0.92
Sepharose–mercurial column	1.07	1.02

[a] The reactions were carried out at pH 5.0 in 0.05 M sodium acetate buffer containing 1 mM EDTA and 10 mM [^{35}S]sulfite under N_2 for 18 hr at 25°. Portions of the [^{35}S]sulfite-treated proteinase were first treated with 0.01 M dithioerythritol at pH 5.5 in 0.05 M sodium acetate buffer for 2 hr at 25° prior to chromatography on the Sephadex G-25 column (see Figure 6). [b] See text and Figure 5 for the preparation of Hg^{2+} free reduced proteinase. [c] Results are expressed as moles of [^{35}S]-sulfite bound per mole of reduced proteinase.

Discussion

The amount of active enzyme (normality) in proteinase solution has been determined by a number of methods, including titration of the SH groups with DTNB and stoichiometric inhibition of the enzyme with $HgCl_2$ (Figure 3) or iodoacetic acid (Figure 4). The results are consistent with the conclusion that the sulfhydryl titer of the proteinase is a direct measure of the amount of active enzyme (normality) present. Of the several procedures employed for the active-site titration, the one based on the reaction of proteinase SH groups with DTNB is the most rapid and convenient.

The zymogen isolated from the streptococcal culture has been shown to be homogeneous by chromatography on ion-exchange columns, N-terminal group analysis, amino acid analysis, and ultracentrifugal studies (Liu and Elliott, 1971). The enzyme derived from the tryptic digestion of the crystalline zymogen has also been shown to be homogeneous by the same criteria (Liu and Elliott, 1971). Both the zymogen and the enzyme have been shown to have one residue of potential half-cystine per mole of protein by amino acid analysis of the performic acid oxidized protein. The present study revealed, however, that activation of the zymogen or the enzyme with a thiol such as DTE results in reduced proteinases exhibiting different specific activities. Further, it has been shown (Table I) that the sole demonstrable difference is in thiol contents and that there is proportionality between activity and thiol content.

Successful separation of active mercaptoproteinase and inactive nonmercaptoproteinase was achieved by the use of a Sepharose–mercurial column originally developed by Sluyterman and Wijdenes (1970) for the purification of active papain (Figure 5). The active proteinase contained one titrable SH group per mole of enzyme and exhibited a maximum

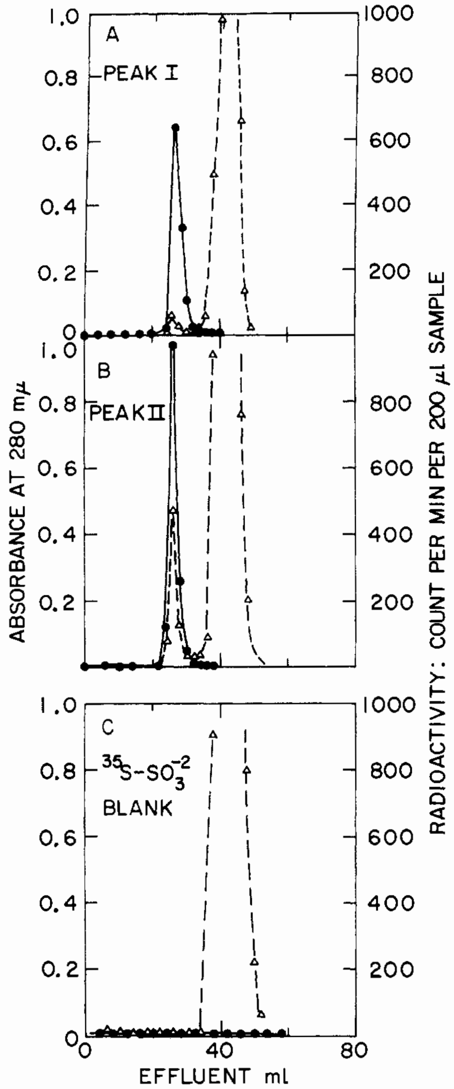

FIGURE 6: Gel filtration of [^{35}S]sulfite treated proteinase on a column of Sephadex G-25 (0.9 × 85 cm) in 0.04 M NaCl and 0.001 M EDTA. Protein (●) was determined by using the optical density at 280 mμ and radioactivity (Δ) as described under Methods: (A) peak I from Sepharose-mercurial column; (B) peak II from Sepharose-mercurial column; (C) [^{35}S]sulfite blank.

specific activity, while the inactive proteinase contained less than 0.05 mol of titrable SH group per mole of enzyme and failed to reveal any enzymic activity. These results are in agreement with the conclusion that the single SH group in streptococcal proteinase is essential for its enzymic activity.

The nature of the inactive proteinase is not clearly known. The data shown in Table II show that the amino acid compositions of the active and inactive proteinases are almost identical except for the content of the half-cysteine. The proteins in the inactive fraction contained 0.4 residue of half-cysteine per mole of protein as determined by the performic acid oxidation procedure.

Since the inactive proteinase was shown by DTNB titration to have less than 0.05 mol/mol of free SH groups, the presence of 0.4 residue of cysteic acid in the performic acid oxidized sample must mean that a portion (40%) of the active-site cysteine exists as a sulfinic (SO_2H) or sulfonic (SO_3H) acid. These derivatives of cysteine would yield cysteic acid upon performic acid oxidation but would not be available for reaction with DTNB or $HgCl_2$. The intermediate sulfenic acid (SOH) is reversible to sulfhydryl upon treatment with an excess of thiol. However, since the enzymic activity of the inactive proteinase cannot be regenerated by treatment with excess thiol, the active-site cysteine presumably does not exist as sulfenium ion.

The question remains as to the nature of the remaining 60% of the inactive proteinase. Amino-terminal residue analysis of the inactive proteinase by the method of Stark and Smyth (1963) showed it to contain 0.85 residue of Glu. Primary sequence studies going on in our laboratory have established (Kortt and Liu, 1973c) the position of the cysteine residue in the streptococcal proteinase to be 56 residues from the N-terminal glutamine. As indicated earlier, the amino acid compositions of the active and inactive proteinases are very similar. These data suggest that the loss of cysteine in a portion (60%) of the inactive proteinase is very unlikely to be due to the autocatalytic cleavage of the peptide including the cysteine residue in the enzyme. One plausible explanation could be the loss of "sulfur" by β elimination from the cysteine residue in the proteinase to yield dehydroalanine during the activation process, which is performed at pH 7.0 ± 0.1 and 37° in the presence of DTE. Further studies will be directed to clarification of this point. Sulfite ions are known to cause the scission of disulfide bonds to form *S*-sulfonates

$$RSSR + SO_3^{2-} \rightleftharpoons RS^- + RSSO_3^-$$

To convert the SH residues of a protein to *S*-sulfonate residues, it is necessary to oxidize the SH to S–S, which may in turn be split by sulfite ions

$$RSSR + SO_3^{2-} \rightleftharpoons RS^- + RSSO_3^- \quad (RS^- \xrightarrow{[O]} RSSR)$$

Exposure of *S*-sulfocysteine to excess thiol will result in the regeneration of RS^-, so that the reaction is reversible.

On the basis of the evidence obtained in this study and the discussion presented above, the inactivation of proteinase with SO_3^{2-} is most unlikely to be due to the formation of proteinase–S–SO_3^-. Since the active proteinase contains only a single SH group per mole of enzyme and there is no disulfide bond present in the molecule, reaction with SO_3^{2-} will not lead to the formation of protein–S–SO_3^-. Moreover, the protein-bound [^{35}S]sulfite was not released even after treatment with excess thiol compounds, which indicates that the [^{35}S]-sulfite is bound to a group other than protein SH.

Sulfite ions are also known to react with carbonyl groups. Activated proteinase, however, is not sensitive to reagents having affinity for carbonyl groups such as phenylhydrazine and NH_2OH. Treatment of activator-free proteinase with these reagents under conditions identical with those used for reaction with Na_2SO_3 resulted in neither inactivation of the enzyme nor binding of reagents to the protein.

Sulfite ions can form covalent bonds by addition to the indole ring (Hesse, 1899). Morihara and Nagami (1969) have suggested the possibility of a reaction of this type in the HSO_3^-–SO_3^{2-} inhibition of papain. The presence of a tryptophan residue near the active site of papain has been implicated from studies of the fluorescence properties of both active and "inactive" papain (Shinitzky and Goldman, 1967; Barel and Glazer, 1969; Weinryb and Steiner, 1970; Sluyterman and DeGraaf, 1970) and has been confirmed by X-ray crystallographic studies (Drenth *et al.*, 1968). However, direct evidence in sup-

port of the binding of sulfite to the active-site tryptophan residue of papain is lacking.

By treating the unreduced streptococcal proteinase with 2-hydroxy-5-nitrobenzyl bromide, Robinson (1970) has shown that a tryptophan residue in the proteinase was modified with a maximum loss of 95% of its potential activity. He proposed that the tryptophan residue modified is near the active site of proteinase. In our present study, we have demonstrated that sulfite binds to the proteinase and inactivates the enzyme. But as yet we do not have evidence to suggest that this inactivation is due to binding of the sulfite to the indole ring of the tryptophan residue in the active site of the enzyme. Peptide(s) containing the [^{35}S]sulfite moiety is being isolated for characterization.

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