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The Reaction of Trypsin with Bromoacetone*

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ABSTRACT: The reaction of trypsin and certain trypsin derivatives with bromoacetone has been investigated. The resultant inactivation of the enzyme, measured by the rate of hydrolysis of ester substrates or by reaction with [^{32}P]diisopropylphosphofluoridate ([^{32}P]DFP), correlated with loss of one histidine residue. Histidine also reacted in diisopropylphosphoryl- (DIP) trypsin but not in L-1-chloro-2-tosylamido-7-amino-2-heptanone (TLCK) trypsin. Experiments were also carried out using [1,3-^{14}C]bromoacetone. A chromatographic procedure, based on the high affinity of trypsin for soybean trypsin inhibitor, was employed to separate active from inactive trypsin in the reaction mixture. The inactivated trypsin contained 1.06 moles of [^{14}C]-acetone more than did the active material and showed a loss of 0.8 residue of histidine when compared with the internal control. Thus inactivation resulted from modification of a single residue of histidine. The radioactive peptides present in the inactive but not in the active bromoactone-treated trypsin contained 1.1 moles of ^{14}C and included histidine-46, while the peptide including histidine-27 contained a negligible quantity of ^{14}C. Bromoacetone was also found to react almost exclusively with histidine-46 in DIP-trypsin. Thus of the three histidine residues present in trypsin, only histidine-46 reacted to a significant extent with bromoacetone. The rate of reaction of histidine-46 in trypsin was comparable to that of the model compound α-*N*-benzoyl-L-histidine methyl ester. Furthermore, the introduction of an acetonyl group into histidine-46 abolished the unusual reactivity of serine-183, thus providing chemical evidence in support of the hypothesis that interaction between these residues is essential for the catalytic function of trypsin.

The involvement of histidine in the catalytic function of trypsin has long been inferred fron the pH dependence of the hydrolysis of specific substrates (Gutfreund, 1955) and from the reaction of the enzyme with *p*-nitrophenyl acetate (Dixon and Neurath, 1957). More recently, Shaw *et al.* (1965) have demonstrated that trypsin will react stoichiometrically with the substrate analog TLCK[1] to form an inactive derivative in which one histidine residue has been alkylated.

Trypsin contains three histidine residues. Two of these, in positions 29 and 46, are brought into close proximity in the linear sequence (Walsh *et al.*, 1964) by virtue of a disulfide bridge which cuts off a segment of the peptide chain containing 15 amino acid residues. A similar "histidine loop" occurs in the amino acid sequence of chymotrypsin (Walsh and Neurath, 1964; Hartley, 1964) and other serine proteases (Hartley *et al.*, 1965) with the two histidine residues located in the same relative position. On the basis of these homologies, it has been suggested that the two histidine residues might have been conserved during evolution in order to fulfill a functional role in enzyme catalysis (Walsh *et al.*, 1964). Bender and Kézdy (1964) have shown that the involvement of two histidine residues might be mechanistically favorable. No functional role has yet been proposed for the third histidine residue in trypsin which does not occur in the homologous enzymes chymotrypsin A or B (Desnuelle and Rovery, 1961).

The single histidine residue that becomes alkylated upon reaction with TLCK is histidine-46 (Shaw and Springhorn, 1967). This residue corresponds to histidine-57 in chymotrypsin, which likewise is singularly alkylated by reaction with the analogous chloromethyl ketone TPCK (Ong *et al.*, 1965). This situation is qualitatively different from ribonuclease where, by use of alkylating agents such as bromoacetate and iodoacetate, either one of two histidine residues of the active site (19 and 112) can become alkylated (Barnard and Stein, 1959; Crestfield *et al.*, 1963). Ribonuclease may represent a special case, however, since the histidine residues of the active center react considerably more rapidly

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[1] Abbreviations used: DIP, diisopropylphosphoryl; DPC, diphenylcarbamyl; TLCK, L-1-chloro-2-tosylamido-7-amino-2-heptanone; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; STI, soybean trypsin inhibitor; PTI, pancreatic trypsin inhibitor; BA-trypsin, bromoacetone-treated trypsin.

with iodoacetate than do model compounds, and are available for reaction at a pH lower than that normally anticipated for histidine (Hendrickson *et al.*, 1965). It was therefore of interest to test the selective reactivity of histidine residues of trypsin with "nonspecific" alkylating agents which bear little structural resemblance to specific substrates.

After preliminary survey of several reagents which included bromoacetate, iodoacetamide, and β-bromopyruvate, our attention was directed toward bromoacetone. This reagent satisfies the requirements for a small uncharged reagent which has no obvious structural similarity to a substrate. Furthermore, experiments with α-*N*-benzoyl-L-histidine methyl ester indicated that the reagent was capable of alkylating histidine at a rate which was similar to the rate of reaction of histidine residues in trypsin. Independent preliminary observations reported by Glick and Koshland (1965), describing the reaction of bromoacetone with chymotrypsin, confirmed our belief in the success of this experimental approach. It was expected, therefore, that bromoacetone would alkylate any "surface" histidine residue in trypsin whether it is a component of the active site or not, in contrast to the "affinity label" TLCK which as already stated reacts exclusively with one of the histidine residues of the active site.

Materials

Trypsin was a twice-crystallized preparation containing 50% $MgSO_4$ obtained from Worthington Biochemical Corp., Freehold, N. J., or was prepared by activation of crystalline trypsinogen (Worthington) by the method of Pechère and Neurath (1957). Enzyme solutions were dialyzed exhaustively against 10^{-3} N HCl and stored at −20°. Determination of protein concentration was based on an $E_{280}^{1\%}$ of 14.4 (Davie and Neurath, 1955).

Soybean trypsin inhibitor and *pancreatic trypsin inhibitor* were Worthington products. Values of $E_{280}^{1\%}$ of 9.09 (Laskowski, 1955) and 8.33 (Kassel *et al.*, 1963) were used for the two proteins, respectively. Twice-crystallized *pepsin* was also obtained from Worthington.

DIP-trypsin was prepared by the method of Cunningham (1954) omitting crystallization. The specific activity of the product was less than 3% of that of the starting material.

DPC-trypsin was prepared essentially as described by Erlanger and Cohen (1963) except that an eightfold molar excess of DPCC was added. The product showed less than 3% of the initial activity. In reactivation experiments, aliquots (0.1 ml) containing 0.5 mg of trypsin were diluted into 0.9 ml of a solution containing 0.1 M $CaCl_2$, 0.1 M benzamidine-HCl, 1 M hydroxylamine-HCl, and 0.2 M Tris-Cl (pH 8.0). After incubation at 25° for 44 hr, activity reached a plateau and reactivation was determined after this period.

TLCK-trypsin was prepared by the method of Shaw *et al.* (1965). The product was further purified by chromatography on a column (2.5 × 32 cm) of SE Sephadex C-50 which had been equilibrated with 0.31 M NaCl–0.005 M citrate (pH 3.0). Elution of the column was at a flow rate of 0.66 ml/min with the same buffer, at 4°.

Diisopropylphosphofluoridate (DFP) was obtained from Merck & Co., Rahway, N. J., and the ^{32}P-labeled material from New England Nuclear, Boston, Mass., as was [1,3-^{14}C]acetone. *Diphenylcarbamyl chloride* was twice-crystallized product of Mann Research Laboratories, Inc., New York, N. Y. *L-1-Chloro-3-tosylamido-7-amino-2-heptanone hydrochloride* was obtained from Cyclo Chemical Corp., Los Angeles, Calif., and was also received as a gift from Dr. Elliott Shaw. *Benzamidine hydrochloride* was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. *N-α-Benzoyl-L-arginine ethyl ester hydrochloride*, *N-α-benzoyl-L-histidine methyl ester*, *L-arginine monohydrochloride*, and *dithioethreitol* were obtained from Cyclo. *2-(N-Morpholino)ethanesulfonic acid* was purchased from Calbiochem., Los Angeles, Calif., and *2-dimethylaminoethanol* from Eastman Organic Chemicals, Rochester, N. Y.

Guanidoethylcellulose (Serva) was purchased from Gallard Schlesinger Chemical Manufacturing Corp., Garden City, N. Y., and Sephadex products from Pharmacia, Piscataway, N. J.

Bromoacetone was synthesized by the method of Levine (1946). The product was stabilized by the addition of a trace of magnesium oxide (Catch *et al.*, 1948) and stored at −20°. All experiments were carried out with freshly redistilled bromoacetone.

Methods

Enzyme Assays. The assay mixture consisted of 0.01 M BAEE, 0.05 M $CaCl_2$, 0.1 M KCl, and 0.01 M Tris-Cl buffer (pH 7.8). Hydrolysis of substrate was followed by continuous titration with 0.1 N NaOH at pH 7.8 and 25° using a pH-stat as described by Bargetzi *et al.* (1963).

Amino acid analysis was performed on a Beckman Spinco Model 120 amino acid analyzer according to Spackman *et al.* (1958). Samples were hydrolyzed with glass-distilled, constant-boiling HCl in evacuated and sealed Pyrex tubes at 104° for 24 hr in the presence of a trace of phenol, except where other conditions are specified. Internal standards were included in samples as described by Walsh and Brown (1962).

Scintillation counting employed a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. The volume of samples was 1 ml and the scintillant solution contained 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 0.375 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene dissolved in 1 l. of dioxane.

Alkylation Conditions. Because the spontaneous hydrolysis of bromoacetone necessitated the continuous addition of base to maintain a constant pH, alkylation was carried out in the reaction vessel of a Radiometer Model TTT1 pH-stat. Reaction mixtures generally contained 0.075 M benzamidine hydrochloride, 0.075 M $CaCl_2$, 0.375 M KCl, 0.29 M bromoacetone, and 2.0×10^{-4} M trypsin. Trypsin was added after the bromoacetone had completely dissolved. Experiments with trypsin were performed between pH 5.5 and 7.5 at intervals of 0.5 pH; with trypsin derivatives all ex-

periments were at pH 6.5. Control experiments to which no bromoacetone had been added were carried out. In some experiments, equivalent concentrations of acetone or of a mixture of hydroxyacetone and KBr were added in place of bromoacetone. All reactions were performed at 40° in the absence of light.

pH was maintained by addition of 1.0 N KOH, with the exception of experiments at pH 7.5, where 3.0 N KOH was used. It was found important that the reaction mixture be adequately stirred to avoid denaturation resulting from localized increase in pH. In some experiments, KCl in the reaction mixture was replaced by 0.375 M Tris-Cl (pH 7.0), and the pH was maintained by the addition of 1 M Tris-Cl (pH 9.5). These experimental variations had no significant affects on the time course of inactivation. For activity measurement, samples were withdrawn from the reaction mixtures and diluted into ten volumes of 2×10^{-3} N HCl at 0°. Aliquots were assayed against BAEE.

Samples for amino acid analysis (1.0 ml) were removed at 30-min and 180-min reaction. They were acidified with 10 μl of 1 N HCl cooled to 4°, centrifuged, and then applied to columns of Sephadex G-25 (2 × 40 cm). Break-through fractions were pooled, dried on a rotary evaporator, and hydrolyzed in preparation for analysis.

[^{32}P]DFP Reaction. Samples of trypsin or bromoacetone-treated trypsin (2.5 mg of protein/ml in 0.05 M $CaCl_2$–0.1 M Tris-Cl, pH 7.2) were treated with one-tenth volume of 0.0125 M [^{32}P]DFP (dissolved in anhydrous *n*-propyl alcohol) for 5 hr at 4°. The reaction products were acidified to pH 3.0 and dialyzed against four changes of 1 l. of 10^{-3} N HCl over a period of 72 hr. Each dialyzed solution was made up to 5 ml, the protein concentration was determined by absorbance measurement, and the relative ^{32}P incorporation was estimated by scintillation counting.

STI Titration. The method used was based on that of Green (1953). Trypsin inhibitor was dissolved in 0.05 M $CaCl_2$–0.5 M Tris-Cl (pH 7.8) (measured at 25°) to give a concentration of about 11 mg/ml. Concentration was determined using the optical factor of 1.1 quoted by Laskowski (1955). Increasing amounts of STI made up in 0.5 ml of buffer were added to samples containing about 1 mg of trypsin, or modified material of equivalent activity, made up in 0.5 ml of 10^{-3} N HCl. The remaining tryptic activity against BAEE was determined on aliquots of 20 or 100 μl. The ordinate intercept of the linear portion of a plot of activity remaining *vs.* milligrams of STI added gave the weight of inhibitor equivalent to a given weight of trypsin, or trypsin derivative.

Alkylation in the Presence of PTI. A solution containing trypsin (5 mg/ml) and PTI (6 mg/ml) was allowed to react with bromoacetone under the previously described conditions at pH 6.5. A control incubation without bromoacetone was also performed. Aliquots of the reaction mixtures were diluted into ten volumes of 0.05 N HCl at 4°. Samples were assayed against BAEE after standing for 90 min. Preliminary experiments demonstrated that under these conditions (pH ~1.6) 90% of the activity present before addition of inhibitor could be recovered.

Alkylation of N-α-benzoyl-L-histidine methyl ester (6×10^{-4} M) was carried out at 40° and pH 6.5. Aliquots of 0.5 ml were removed and diluted into 1 ml of L-arginine (0.4 μmole/ml) in 0.1 N HCl at 4°. Samples were dried on a rotary evaporator, redissolved in 1 ml of 1 N HCl, and taken to dryness again before hydrolysis and amino acid analysis. Histidine recoveries were calculated on the basis of the arginine value expected. Addition of bromoacetone to the internal arginine standard did not cause destruction of arginine.

Alkylation with [1,3-^{14}C]Bromoacetone. SYNTHESIS OF LABELED BROMOACETONE. The procedure of Levine (1946) was employed but with a 50-fold reduction in scale using [1,3-^{14}C]acetone and with the following modifications. After separation of the organic phase containing crude bromoacetone the aqueous phase was reextracted with four 0.6-ml portions of unlabeled bromoacetone. All these bromoacetone-containing extracts were pooled. The product was distilled without prior drying. On redistillation, a product boiling at 35.5–37.5° at 12 mm was obtained. Based on radioactivity, the yield was 48% and the specific activity of the product 55.7×10^3 dpm/μmole.

Reaction of Trypsin and DIP-trypsin with [^{14}C]Bromoacetone. The trypsin used (Worthington lot 6EA) had a specific activity toward BAEE of 50.8 μequiv/min per mg. Reaction mixtures contained benzamidine hydrochloride (0.075 M), calcium chloride (0.075 M), 2-(*N*-morpholino)ethanesulfonic acid (0.005 M), bromoacetone (0.29 M), and trypsin (or DIP-trypsin) (12.5 mg/ml) in a final volume of 20 ml. Trypsin or its DIP derivative was added after all bromoacetone had dissolved and the pH had been adjusted to pH 6.5. The pH was adjusted and maintained with 10% (v/v) 2-dimethylaminoethanol in a pH-Stat. The reaction was carried out at 40° and pH 6.5 in the dark and was terminated after 1 hr by cooling the reaction mixture to 4° and adjusting the pH to 3.0 with 1 N HCl. The solution was centrifuged at 18,000 rpm for 10 min to remove a slight turbidity and the supernatant was applied to a column (2.5 × 80 cm) of Sephadex G-25 (coarse) which had previously been equilibrated with 10^{-3} N HCl at 4°. Fractions in the break-through peak monitored by absorbance at 280 mμ were pooled and lyophilized. Recoveries of 90% for trypsin and 86% for DIP-trypsin were obtained, based on 280-mμ absorbance.

Separation of Active and Inactive BA-trypsin. In order to compare the active and inactive components in the reaction mixture produced by the limited reaction of trypsin with bromoacetone a method for their separation was developed, based on the different affinities of the active and inactive components for STI.

It was found in preliminary experiments that trypsin was not retained on GE-cellulose, whereas little or no protein appeared in the break-through fraction when an excess of STI had been added to the trypsin. However, it was necessary to maintain an ionic strength of at least 0.1 in order to prevent precipitation of the complex. Addition of calcium to the buffer was designed to minimize proteolysis by any trypsin not retained in the complex. The following procedure was used to separate the products of partial reaction between bromoacetone

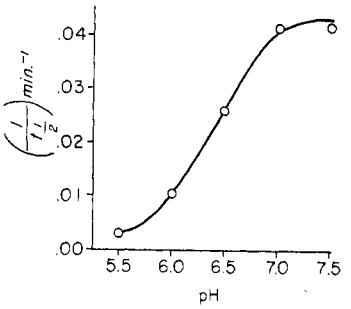

FIGURE 1: pH dependence of the inactivation of trypsin with bromoacetone. Data are uncorrected for the hydrolysis of bromoacetone. Reaction conditions are described in the text.

and trypsin into those components which would form a highly associated complex with STI and those which complexed weakly or not at all with the inhibitor.

Lyophilized BA-trypsin (220 mg) was added to a solution containing STI (135 mg) dissolved in 25 ml of 0.07 M Tris-Cl buffer (pH 7.8) containing 0.01 M $CaCl_2$. The solution was readjusted to pH 7.8 (measured at 4°) by the addition of 1 M Tris (about 0.2 ml) and applied to GE-cellulose.

GE-cellulose (Serva lot A1585, 0.38 mequiv/ g) was washed with 0.5 N NaOH, water, and 0.5 N HCl, and then with water until the effluent was neutral. The absorbant was next equilibrated with 0.07 M Tris-Cl (pH 7.8) containing 0.01 M $CaCl_2$. Buffer pH was measured at 25°, but the equilibration and operation of columns were at 4°. Columns (1.5 × 25.5 cm) were packed under 5 psi and were operated at a controlled flow rate of 1.2 ml/ min.

Mixtures of BA-trypsin and STI were chromatographed on GE-cellulose using a single stepwise change in ionic strength. Fractions of 5 ml (25) were collected by eluting with the buffer used for equilibration (fraction I). An additional 20 fractions were eluted with 0.07 M Tris-Cl (pH 7.8) containing 0.01 M $CaCl_2$ and 0.75 M NaCl. Material absorbing at 280 mμ which passed through the column in the starting buffer, but which was eluted by the stepwise increase in salt concentration (tubes 33–38), is termed fraction II. Fractions I and II were pooled separately as indicated, dialyzed against 10^{-3} N HCl, and lyophilized.

Fraction I contained bromoacetone-inactivated trypsin contaminated with autolysis products of trypsin. Fraction II consisted of active bromoacetone-treated trypsin together with STI. The following chromatographic procedure was used to separate these contaminants from fractions I and II.

Purification of Active and Inactive Trypsin. SE Sephadex C-50 was equilibrated with 0.005 M citrate buffer (pH 3.0) containing 0.30 M NaCl. A column was packed (1.5 × 27 cm) without pressure at a controlled flow rate of 0.32 ml/min. Subsequent operation was at the same flow rate of 4°.

Fraction I (125 mg) or II (180 mg) was dissovled in 5.0 ml of the pH 3.0 buffer and adjusted to pH 2.7 with 1 N HCl. Elution was carried out with the same buffer. The contents of the tubes shown in Figure 5 were pooled after chromatography of I and II, dialyzed against 10^{-3} N HCl, and lyophilized. The yield from fraction I of "inactive trypsin" was 96 mg and from fraction II of "active trypsin" was 64 mg. The products of the reaction of bromoacetone with DIP-trypsin, after passage through Sephadex G-25, were similarly chromatographed on SE Sephadex.

Isolation of Peptides

Enzymatic Digestion. Samples of each trypsin derivative (50 mg) dissolved in 5% (vol/vol) formic acid (5 ml) were incubated with pepsin (1 mg) at room temperature for 14 hr in the presence of toluene. The digest was then lyophilized.

Peptide Chromatography. Pyridine acetate buffers of pH 3.1 and 5.0 with respective pyridine concentrations 0.2 and 5.0 M were prepared according to Schroeder *et al.* (1962). Chromatography was carried out on columns of SE Sephadex C-25 (1.5 × 29 cm) previously equilibrated with buffer at pH 3.1. Elution was performed with a gradient linear in pyridine concentration employing 200 ml each of pH 3.1 and 5.0 buffers connected by a siphon. The outflow of buffer from the column was controlled with a peristaltic pump set to deliver 0.18 ml/min. Fractions of 1.9 ml were collected.

The lyophilized peptic digests were suspended in 3 ml of pH 3.1 buffer, adjusted to pH 2.5 with 5.7 N HCl, made up to 5 ml, and centrifuged to remove a small amount of insoluble material. The supernatant was applied to a column of SE Sephadex with two 1-ml portions of pH 3.1 buffer. Elution with pH 3.1 buffer was continued until 30 fractions had been collected, when the gradient was started.

Aliquots of 100 μl of alternate fractions were taken for ninhydrin analysis (Schroeder and Robberson, 1965) and 200 μl for scintillation counting. Radioactive peptides were pooled as indicated in Figure 2 and were brought to dryness in a rotary evaporator.

Reduction and Alkylation. Peptides E and F (Figure 6) were dissolved in 1.0 ml of 8 M urea, prepared freshly from recrystallized material and containing 0.05 M *N*-ethylmorpholine (pH 8.5). Two 0.15-ml additions of 0.05 M aqueous dithioethreitol (Cleland, 1964) were made over a 1-hr period at 25°. Alkylation was accomplished by addition of 0.6 ml of 1 M iodoacetate adjusted to pH 8.5 and allowing the solution to stand for an additional 0.5 hr at 25°. The reaction mixture was diluted with 1 ml of 0.2 M pyridine acetate buffer (pH 3.1), adjusted to pH 2.5, and made up to 5 ml with water. The mixture was then applied to SE Sephadex for chromatography under the previously described conditions.

Results

Reaction Conditions. The choice of conditions for the alkylation of trypsin was determined by the stability of the enzyme, the rate of reaction, and the solubility and stability of the alkylating agent. For a preliminary survey it was highly desirable that the reaction should approach completion within a period of a few hours. This was achieved by using a large molar excess (1400-fold) of reagent and an elevated temperature (40°). Trypsin under-

TABLE I: Reaction of Bromoacetone with Trypsin at pH 6.5.[a]

	Reaction Time			
	30 min		180 min	
Amino Acid	Control	Bromo-acetone	Control	Bromo-acetone
Lysine	12.6	13.1	13.0	12.3
Histidine	2.7	2.3	2.7	1.8
Arginine	1.8	1.8	1.8	1.7
Aspartic acid	21.0	21.9	21.0	21.1
Threonine	9.1	9.5	8.8	9.3
Serine	27.2	27.7	27.9	27.5
Glutamic acid	14.0	14.6	13.8	14.1
Proline	7.8	8.0	7.7	7.9
Glycine	25.2	25.5	24.1	25.2
Alanine	14.0	14.0	14.0	14.0
Valine	14.8	14.4	14.4	14.1
Methionine	1.7	1.6	1.5	1.5
Isoleucine	13.5	13.5	13.2	12.9
Leucine	13.5	13.1	13.4	13.1
Tyrosine	9.1	9.1	8.8	8.7
Phenylalanine	3.0	3.0	3.0	3.1

[a] Analytical values were related to 14 moles of alanine. Hydrolysis was for 22 hr in the absence of phenol.

goes rapid autolysis at this temperature but the competitive inhibitor benzamidine greatly diminishes autolysis without preventing inactivation of the enzyme by bromoacetone. Controls showed less than 10% inactivation during a period in which the activity of trypsin treated with bromoacetone had fallen to 50% of the control value.

pH Dependence. The pH dependence of inactivation is shown in Figure 1. Half-time of inactivation was used as an index of relative reaction rates. The data obtained do not represent rate constants as the inactivation did not follow first-order kinetics owing, presumably, to hydrolysis of bromoacetone. Although a precise pH value cannot be assigned, the results suggest that the rate of inactivation of trypsin is controlled by reaction of a residue, or residues, ionizing between pH 6 and 7.

Analysis. Amino acid analysis of reaction mixtures at pH 6.5, 7.0, and 7.5 containing bromoacetone, when compared to controls at the same pH, indicated that the loss of approximately one histidine residue had occurred in a period of 3 hr. The data for the experiment at pH 6.5 are given in Table I. While 0.9 residue of histidine disappeared after 180-min reaction, no other residue (with the exception of lysine which will be discussed later) was significantly diminished, when compared to the appropriate control.

Analysis of the reaction mixture at pH 7.5 indicated the loss of about five residues of lysine in 180 min, in addition to the disappearance of histidine. To avoid extensive modification of lysine, all subsequent experiments were carried out at pH 6.5.

The time course for the inactivation at pH 6.5 is shown in Figure 2. It can be seen that under the conditions employed, inactivation was not complete. This could result either from the formation of partially active enzyme or from a mixture of fully active and fully inactive species.

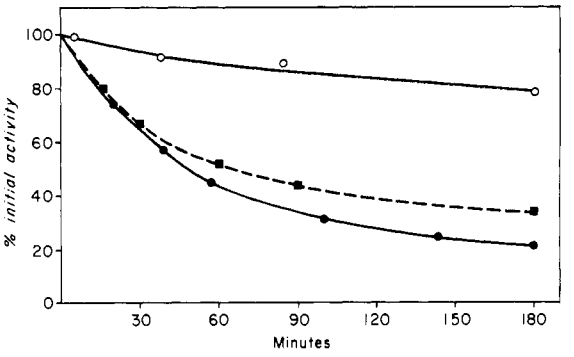

FIGURE 2: Time course for the reaction of bromoacetone with trypsin and with benzoylhistidine methyl ester in the presence of benzamidine at pH 6.5 and 40°. (●) Tryptic activity measured against BAEE bromoacetone present. (○) BAEE activity in control containing no bromoacetone. (■) Histidine remaining in benzoylhistidine methyl ester determined by amino acid analysis following hydrolysis.

[^{32}P]*DFP Reaction and STI Titration.* Table II shows the results obtained when trypsin, which had been treated for 3 hr at pH 6.5 with bromoacetone, was subsequently allowed to react with [^{32}P]DFP until the remaining activity was abolished. The proportion of active sites determined by ^{32}P incorporation (26.6%) is in agreement with that obtained by rate assay (23.3%). It can, therefore, be concluded that inactivation of trypsin with bromoacetone does not result in the formation of partially active trypsin. Furthermore, DFP does not react with trypsin which had been inactivated by bromoacetone.

Partially inactivated trypsin showed the relative combining power with STI anticipated from the rate assay, as indicated in Table III. Similar data were also obtained with PTI. Thus, loss of activity toward BAEE is paralleled by loss of the ability to react with DFP and with the ability to form a highly associated complex with trypsin inhibitors.

Alkylation of Trypsin in the Presence of PTI. Trypsin activity can be measured in the presence of protein inhibitors if the complex is permitted to dissociate at low pH (Green, 1953). When an aliquot of dissociated complex is added to an assay mixture, the substrate competes effectively with the inhibitor to prevent re-formation of

TABLE II: Reaction of Modified and Native Trypsin with [^{32}P]DFP.

	Sp Act. (μequiv/min mg)	Rel Act. (%)	Cpm/mg of Protein	Rel ^{32}P Incorp (%)
Trypsin	47.6	100	40,825	100
BA-trypsin	11.1	23.3	10,844	26.6

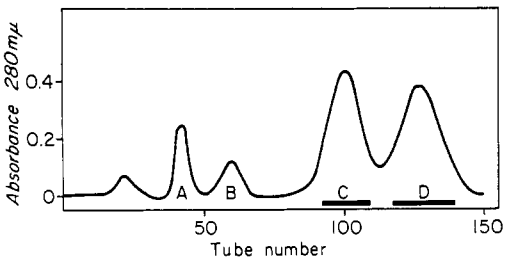

FIGURE 3: Chromatography of TLCK-trypsin on SE Sephadex eluted with 0.31 M NaCl–0.005 M sodium citrate (pH 3.0). The fractions indicated in peaks C and D were pooled. Both gave the amino acid composition expected for TLCK-trypsin.

the complex. This technique was applied to the measurement of the activity of trypsin in the presence of PTI in order to determine whether PTI could protect trypsin against inactivation by bromoacetone.

When trypsin was incubated with bromoacetone for 3 hr in the presence of an excess of PTI, the activity recoverable on acid dissociation was 90% that of a control in which bromoacetone was omitted. The control showed no loss of recoverable activity during the 3-hr time course of the experiment. Under parallel conditions, when PTI was omitted and bromoacetone present, only 25% of the initial activity remained after 3-hr. Pancreatic trypsin inhibitor, therefore, provides almost complete protection against inactivation by bromoacetone.

Alkylation of Trypsin Derivatives. Trypsin derivatives were treated as described above at pH 6.5. Amino acid analysis of DIP-trypsin and DPC-trypsin at 30 and 180 min showed consistent disappearance of only two amino acids, histidine and lysine. The extent of reaction of histidine is summarized in Table IV.

Use of inactive trypsin derivatives eliminates the uncertain effects of autolysis on amino acid composition. From the reaction of DIP-trypsin, DPC-trypsin, and TLCK-trypsin, it was possible to establish that a limited amount of lysine had reacted. This was within the range 0.4–1.0 residue over a 3-hr period. Examination of Table I indicates that some reaction of lysine (0.7 residue/mole) may have occurred in unmodified trypsin.

Partial reactivation of DPC-trypsin could be achieved with alkaline hydroxylamine. As reactivation was not complete, presumably due to the autolysis of trypsin, the values obtained for the extent of reactivation are not strictly quantitative. However, it is of interest that reactivatability observed followed a similar time course as inactivation of unmodified trypsin. This is indicated in Table IV.

TABLE III: The Titration of Trypsin and Bromoacetone-Treated Trypsin with STI.

	Sp Act. (μequiv/min mg)	Rel Act. (%)	mg of STI = 1 mg of trypsin	Rel Inhibn (%)
Trypsin	48.0	100	0.98	100
BA-trypsin	14.4	30	0.28	29

TABLE IV: Residues of Unreacted Histidine.[a]

Time	0 min	30 min	180 min
Trypsin	2.7	2.3 (58)	1.8 (26)
DIP-trypsin	2.9	2.2	1.9
DPC-trypsin	2.8	2.4 (57)	2.1 (15)
TLCK-trypsin	2.05	2.0	2.05

[a] Summary of the data obtained for the reaction of trypsin and derivatives with bromoacetone at pH 6.5 and 40°. Figures in parentheses represent per cent initial activity remaining for trypsin, or per cent of control activity regained for DPC-trypsin.

Before examining the reactivity of TLCK-trypsin toward bromoacetone, a preparation of TLCK-trypsin was freed of autolysis products by chromatography on SE Sephadex. Figure 3 shows the elution pattern obtained. As both peaks C and D gave analyses corresponding to that expected for TLCK-trypsin, material from each of these peaks was treated separately with bromoacetone. Neither showed any detectable loss of histidine from the two remaining residues.

The reason for the existence of two distinct chromatographic species of TLCK-trypsin is unknown. One possibility is that loss of the tosyl group of TLCK as *p*-toluenesulfonamide (Petra *et al.*, 1965) gave rise to a new chromatographic species. Unmodified trypsin gave a single active peak when chromatographed in this system.

Alkylation of N-α-Benzoyl-L-histidine Methyl Ester. The time course of alkylation is indicated in Figure 2. The concentration of benzoylhistidine methyl ester (6×10^{-4} M) was equivalent to the total concentration of histidine in trypsin at the concentration (2×10^{-4} M) employed in other experiments. Reduction in area of the histidine peak (at 48 ml) on the short column of the amino acid analyzer was accompanied by the appearance of a peak at 26 ml and a much smaller peak at 35 ml. In a protein hydrolysate, these components would emerge with tryptophan and lysine, respectively.

The time course of the reaction of the model compound with bromoacetone was similar to the time course of the inactivation of trypsin. Thus, the rates of reaction in the two cases are probably not greatly different.

Alkylation with [1,3-^{14}C]Bromoacetone. SEPARATION OF ACTIVE AND INACTIVE BROMOACETONE-TREATED TRYPSIN. To locate the site at which reaction of bromoacetone with trypsin caused inactivation, alkylation was carried out using labeled reagent. As a control for the experiment active trypsin was separated from inactive material contained in the same reaction mixture. The separation was based on the high affinity of active trypsin for STI, a property which has been shown to be abolished or sub-

TABLE V: Properties of Active and Inactive Material Separated from a Reaction Mixture of Trypsin and [1, 3-^{14}C]Bromoacetone.

	Sp Act. (μequiv/min mg)	Residues of [^{14}C]-Acetone/Mole	Δ Residues of [^{14}C]-Acetone/Mole
BA-trypsin (active)	53.3	1.52	
BA-trypsin (inactive)	0.8	2.58	1.06

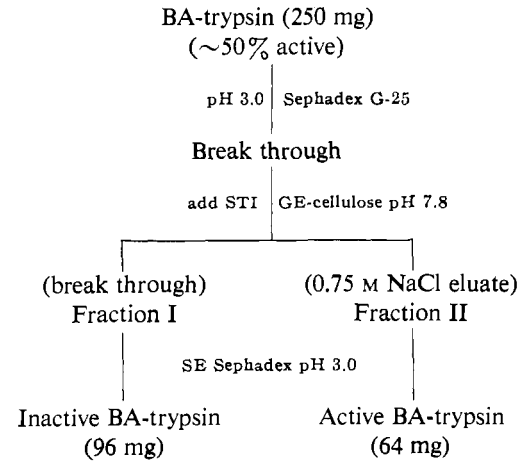

FIGURE 4: Separation of the active and inactive products of the reaction between [^{14}C]bromoacetone and trypsin. For experimental details see the text.

stantially reduced by reaction of trypsin with bromoacetone. The charge of the enzyme is positive at neutral pH while the charges of both the enzyme–inhibitor complex and inhibitor are negative (Laskowski, 1955). Thus, a simple chromatographic separation is possible. To clarify discussion, the methods of separation and purification of active and inactive bromoacetone-treated trypsin are summarized in Figure 4.

The first stage in the purification consisted of the removal of reagent from the reaction mixture by chromatography on Sephadex G-25. A mixture was recovered which contained inactivated trypsin, active trypsin, and some autolysis products. After addition of STI to this mixture, the components which failed to be absorbed by GE-cellulose at an ionic strength of 0.1 were termed fraction I. This contained inactive trypsin, together with some autolysis products. Both the active trypsin, which was complexed with STI, and the excess inhibitor were absorbed at ionic strength 0.1 but were eluted (fraction II) by increasing the ionic strrength to 0.85. Further chromatography of I and II was carried out on SE Sephadex in order to purify the active and inactive bromoacetone-treated trypsin.

Figure 5A shows the elution pattern obtained when fraction II was chromatographed on SE Sephadex at pH 3.0. The low pH at which chromatography was carried out was sufficient to dissociate the trypsin–inhibitor complex to a level where chromatographic separation could be obtained. All of the enzyme in the peak was fully active, as evidenced by the specific activity which was constant across the peak. This material was "active bromoacetone-treated trypsin." The STI which was present in fraction II emerges considerably later than trypsin and its elution is not shown in Figure 5.

Chromatography of fraction I is illustrated in Figure 5b. The major component appeared similar in chromatographic behavior to the active material obtained from fraction II but was almost completely devoid of activity. This was inactive bromoacetone-treated trypsin. A break-through peak was also observed in this elution pattern. The material in this first peak was inactive and passed slowly through dialysis tubing. It was, therefore, of substantially lower molecular weight than trypsin and probably arose by autolysis.

The chromatographic system employing SE Sephadex accomplished both the removal of STI from active bromoacetone-treated trypsin and the removal of autolysis products from inactive bromoacetone-treated trypsin. After purification in this system, the properties of both active and inactive material could be compared.

PROPERTIES OF ACTIVE AND INACTIVE BA-TRYPSIN. Table V summarizes the specific activity and radioactivity of active and inactive trypsin separated on the basis of their different affinities toward STI. The specific activity of the active bromoacetone-treated material compares favorably with that of the trypsin used as starting material which was 50.8 μequiv/min per mg. This small increase in specific activity (5%) probably results from the removal of inactive material rather than

TABLE VI: Amino Acid Analysis of the Active and Inactive Products Separated from a Reaction Mixture of Trypsin and [^{14}C]Bromoacetone.

	Active BA-trypsin	Inactive BA-trypsin
Lysine	12.0	12.2
Histidine	*2.8*	*2.0*
Arginine	2.1	2.2
Aspartic acid	22.7	23.4
Threonine	9.9	10.0
Serine	29.4	28.8
Glutamic acid	14.7	15.1
Proline	7.9	8.1
Glycine	25.0	24.9
Alanine	14.0	14.0
Cystine (½)	11.2	11.6
Valine	14.2	13.8
Methionine	2.0	2.0
Isoleucine	13.9	14.0
Leucine	14.1	14.0
Tyrosine	10.0	10.0
Phenylalanine	2.9	3.2

TABLE VII: Histidine Loop Peptides Obtained from Trypsin Inactivated with [^{14}C]Bromoacetone.

Peptide	E_1	F_1	Theory	E_2	F_2	Theory
CM-cysteine	1.00	1.00	1	1.00	1.00	1
Aspartic acid	0.95	0.94	1	0.12	0.10	
Serine	1.80	0.75	2	1.94	1.50	2
Glutamic acid				0.95	1.01	1
Glycine	2.94	3.05	3	1.15	1.18	1
Alanine				1.88	2.06	2
Valine				1.08	0.84	2[a]
Isoleucine				0.91	0.96	1
Leucine	0.94	1.00	1			
Tyrosine	0.86	0.91	1	0.91	0.99	1
Phenylalanine	0.88	1.02	1			
Lysine				0.97	0.95	1
Histidine	*0.88*	*0.95*	*1*	*0.18*	*0.20*	*1*
"BA-histidine"	Absent	Absent		Present	Present	
Moles of [^{14}C]-acetone	0.05	0.07		1.05	1.09	
Yield (%)	10.1	29.3		16.9	32.8	

[a] See text for discussion of the valine content of the peptides.

as a result of chemical modifications. The low level of activity in the inactive trypsin probably results from the presence of a small amount of active trypsin. Amino acid analysis of both active and inactive bromoacetone-treated material (Table VI) indicated that they had similar composition, except for a clear difference in histidine content, of about 0.8 residue.

The difference in radioactive groups incorporated between active and inactive derivatives of 1.06 residues/mole (Table V) indicates that inactivation accompanies reaction of one residue on the protein with approximately 1 mole of [^{14}C]bromoacetone to give a labeled derivative. Taken together with the observed loss of histidine in the inactive trypsin, it can be concluded that inactivation accompanies modification of histidine by bromoacetone, the primary product being a monosubstituted derivative.

Analysis of the basic amino acids in hydrolysates of both active and inactive bromoacetone-treated trypsin was performed with a 20-cm column instead of the 10-cm column routinely employed. A small peak, of unidentified origin, emerged as a shoulder following lysine. The partial separation of this derivative, whose identity has not been determined, makes the lysine values recorded in Table VI slightly lower than those in other experiments where basic amino acids were separated in the usual 10-cm column.

ISOLATION OF PEPTIDES. The elution patterns of radioactive peptides obtained from the peptide digests of active and inactive bromoacetone-treated trypsin are shown in Figure 6. While peaks A–D are common to both active and inactive material, peaks E and F are present, to a significant extent, only in the inactive trypsin. For this reason, only the "difference peptides" E and F were examined further. Following reduction and alkylation E and F gave the patterns shown in Figure 7 when rechromatographed in the *same* system that had been used for their initial separation. The absence of any counts or ninhydrin-positive material in the fractions where the peptides appeared on first chromatography indicates that reduction and alkylation were complete. While peptides F1 and E1 (Figure 7) appeared to be chromatographically identical peptide F2 emerged slightly later than E2, indicating that these peptides were not identical. It was also apparent that F1 and E1 contained essentially no radioactive material while F2 and E2 were radioactive.

The composition of peptides E1 and F1 (Table VII) is uniquely consistent with the sequence: Asn,Ser,Gly,-Tyr,His(29),Phe,CM-Cys-Gly-Gly,Ser,Leu (Walsh *et al.*, 1964). No new histidine derivatives were detected and the expected value for histidine was obtained. In view of these observations, and the absence of significant radioactivity associated with peptides F1 and E1 in Figure 7, the values of 0.05 and 0.07 μmole of [^{14}C]acetone incorporated may represent an overestimate, or a maximum value for the reaction of histidine-29.

The data obtained for peptides F2 and E2 are consistent with the composition of the peptide: Val,Val,Ser,-Ala,Ala,His(46),CM-Cys-Tyr,Lys,Ser,Gly,Ile,Gln except for the low histidine value and the presence of a peak appearing on the analyzer in the position at which tryptophan normally emerges. This behavior is analogous to that observed when α-*N*-benzoyl-L-hisitidine methyl ester reacts with bromoacetone where loss of histidine was accompanied by the appearance of a new derivative in the region of tryptophan. The level of radioactivity observed in peptides E2 and F2 is close to 1 mole of [1,3-^{14}C]acetone/mole of peptide.

The analyses of 22-hr hydrolysates of E2 and F2 do

TABLE VIII: Amino Acid Analysis of DIP-Trypsin and DIP-trypsin after Treatment with Bromoacetone.

	DIP-trypsin	DIP-trypsin Treated with BA	Theory[a]
Lysine	13.9	13.3	14
Histidine	*2.88*	*2.13*	*3*
Arginine	1.73	1.69	2
Aspartic acid	21.8	22.4	22
Threonine	9.69	9.83	10
Serine	31.0	30.2	33
Glutamic acid	13.8	14.0	14
Proline	7.15	7.07	8
Glycine	25.2	24.8	25
Alanine	14.0	14.0	14
$^1/_2$ Cystine	11.5	10.9	13
Valine	13.3	14.0	17
Methionine	1.81	1.43	2
Isoleucine	13.2	13.6	14
Leucine	14.0	13.2	14
Tyrosine	9.54	9.64	10
Phenylalanine	3.06	3.18	3

[a] This composition is based on that of trypsinogen (Walsh and Neurath, 1964) amended by Mikeš *et al.* (1966). The activation peptide (Val-Asp_4-Lys) and one serine have been subtracted.

not indicate the reason for their slightly different chromatographic behavior. However, after hydrolysis for 100 hr amino acid analysis showed that E2 contained 2.09 and F2 contained 1.01 residues of valine, calculated on the basis of 2.00 residues of alanine. It is known that the Val,Val sequence occurring at the N terminus of the histidine(46) peptide is resistant to hydrolysis. Thus, only half the valine present appears after 22-hr hydrolysis of E2. However, F2 contains only one residue of valine and must result from a peptic split of the Val–Val bond present in the N-terminal dipeptide of E2.

DIP-TRYPSIN. Reaction conditions and the chromatography of the reaction products of bromoacetone-treated DIP-trypsin were the same as those described for the trypsin–bromoacetone product except that the treatment with STI and chromatography on GE-cellulose were omitted, because DIP-trypsin fails to complex with STI (Green, 1953). Amino acid analysis of the starting material and the partially reacted product, shown in Table VIII, indicate the loss of 0.75 residue of histidine in the reaction. On digestion of the product with pepsin, and chromatography on SE Sephadex, the elution pattern showed peaks of radioactivity in the same positions as those obtained from the inactive reaction products of trypsin and bromoacetone (Figure 5b).

Following reduction and alkylation, peptides were obtained as described above for the inactive trypsin–bromoacetone product. Essentially identical results were obtained both in the location of peptides and the distribution of radioactivity between them. Some analytical data of the peptides are given in Table IX. In composition and distribution of radioactivity they were similar to those obtained from inactive trypsin (see Table VII), except that a higher recovery of histidine was obtained in both peptides originating from hisitidine-46. This was to be anticipated because in this experiment "active" and "inactive" material could not be separated.

TABLE IX: "Histidine Loop" Peptides Obtained from DIP-trypsin Partially Reacted with [^{14}C]Bromoacetone.

Peptide	E_1	F_1	E_1	F_2
Histidine	0.86	0.92	0.26	0.30
"BA-histidine"	Absent	Absent	Present	Present
Moles of [^{14}C]-acetone	0.06	0.06	0.58	0.64
Yield (%)	7.0	14.7	11.0	19.2

Discussion

The reactivities of an amino acid side chain in proteins may differ significantly from those of model compounds. For this reason, the change in amino acid composition following the reaction of trypsin or trypsin derivatives with bromoacetone was considered to be a more reliable guide in a preliminary identification of the modified amino acid residues than the use of amino acid mixtures or model compounds. This method of analysis has the disadvantage that small changes in amino acid residues present in high proportions or the modification of tryptophan residues may not be detected. Actually, the only residues which were significantly affected were histidine and lysine. Since Fraenkel-Conrat *et al.* (1949) and others (Terminiello *et al.*, 1955, 1958; Sri Ram *et al.*, 1954) had already shown that most of the lysine residues in trypsin may be acylated without loss of activity, histidine was considered to be the most likely residue the loss of which should be related to inactivation. In fact, the observed pH dependence of inactivation by bromoacetone suggested that the rate-controlling group with a pK of pH 6–7 corresponds more closely to the ionization of a histidine than a lysine residue. The observed correlation of the loss in histidine with inactivation added support to this interpretation.

The addition of benzamidine to the reaction mixture of bromoacetone and trypsin was necessary to stabilize the enzyme, but raises the question as to whether this competitive inhibitor influences the course of the reaction in any significant way. Inagami (1965) has reported that the rate of alkylation of histidine in trypsin by iodoacetamide was increased sixfold in the presence of methylguanidine as compared with the native enzyme and 14-fold when compared with the rate in the presence

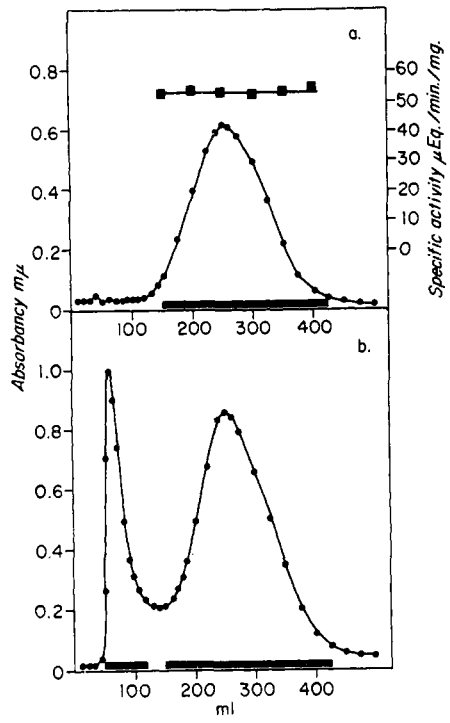

FIGURE 5: Chromatography on SE Sephadex of active and inactive components obtained from a reaction mixture containing [^{14}C]bromoacetone and trypsin. (a) Elution pattern obtained from the active component, fraction II. STI was not eluted in the part of the chromatography shown. (b) Elution pattern obtained from the inactive products, fraction I. The contents of the tubes indicate by heavy lines were pooled.

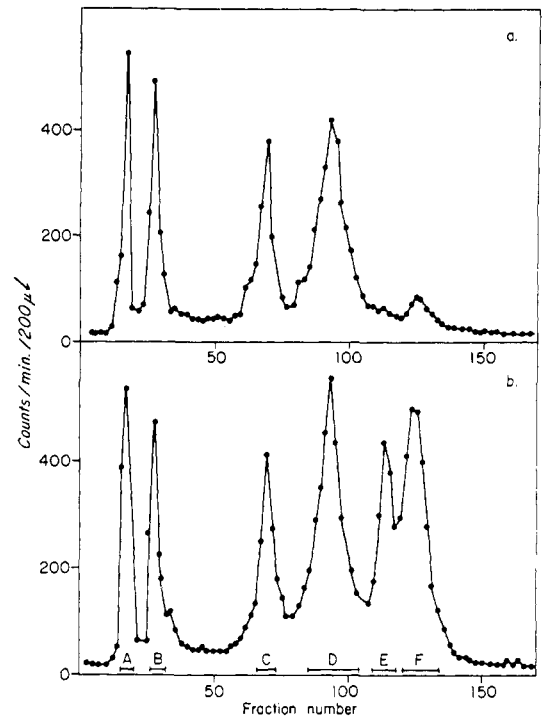

FIGURE 6: Chromatography of peptic digests of (a) active and (b) inactive components isolated from the reaction mixture between [^{14}C]bromoacetone and trypsin. Only the "difference" peptides E and F were examined further.

of butylguanidine. While the effects of benzamidine on the rate of inactivation by bromoacetone have not been investigated in detail in the present work, preliminary evidence suggests that the inactivation rate did not appear to depend significantly on the inhibitor used and that similar inactivation rates were obtained in the presence of benzamidine, methylguanidine, and benzoyl-L-arginine, respectively. No comparative measurements of the rates of inactivation were made in the presence and absence of competitive inhibitors, because of the instability of the enzyme without added inhibitor. Although small competitive inhibitors do not prevent inactivation of trypsin by bromoacetone, pancreatic trypsin inhibitor was found to do so. This relatively small protein has a tightly folded primary structure (Kassell and Laskowski, 1965), and it seems likely that it is in contact with trypsin over a rather limited surface area which would include the active site. Thus prevention of inactivation of trypsin by bromoacetone in the presence of pancreatic inhibitor indicates that the residue or residues involved in inactivation, presumably histidine, are in the proximity of the active site.

The fact that reaction of TLCK-trypsin with bromoacetone does not involve a loss of histidine suggests that the residue or residues normally available to this reagent in the native enzyme are blocked in the TLCK protein (Table IV). This finding leads to the conclusion that any histidine residue which reacts with bromoacetone must be located close to the active center. Since it has already been shown that histidine-46 reacts exclusively with TLCK (Tomášek *et al.*, 1965; Shaw and Springhorn 1967) and that histidine-29 is relatively close, it seemed plausible that one or the other of these residues rather than histidine-79 is involved in the reaction with bromoacetone. This assumption receives additional support from consideration of conformational homology between chymotrypsin and trypsin (Neurath *et al.*, 1968). Thus if the polypeptide chain of trypsin is folded according to the three-dimensional model recently proposed for chymotrypsin by Matthews *et al.*, (1967), histidine-79 is found to be so remote from the active center that it cannot be involved in enzyme function.

The analytical data given in Table IV are in accord with the view that the alkylation of one histidine residue suffices to cause enzyme inactivation. The stoichiometric relationship does not exclude the possibility that partial alkylation of two different histidine residues may in fact be involved in the inactivation reaction. Unequivocal proof, therefore, required the identification of the alkylated histidine residue(s) in the linear sequence of the protein by methods involving labeling, protein degradation, and peptide isolation.

Since under the experimental conditions employed the reaction of trypsin with bromoacetone did not go to completion, it was necessary to separate reacted (inactive) and unreacted (active) trypsin from each other. A chromatographic technique, based on the capacity of active trypsin to form a complex with soybean trypsin inhibitor, was developed for this purpose. After a subsequent purification step, the active and inactive components could be compared on the basis of their reac-

tivity, ^{14}C incorporation, and amino acid composition. If modification of a single residue was responsible for inactivation, it would be predicted that there should be a difference of 1 mole of ^{14}C incorporation (assuming monosubstitution) and a corresponding difference in histidine content between active and inactive species.

The basis of this experimental approach was the observation that trypsin loses its ability to form a highly associated complex with STI when treated with bromoacetone. While trypsin is a strongly basic protein, both STI and the STI–trypsin complex have isoelectric points below neutrality (Laskowski, 1955). It was therefore possible to separate inactivated material, with a similar net charge to that of trypsin, from both complex and free inhibitor simply by passing the mixture through an anion exchanger. The subsequent chromatographic step, using SE Sephadex, was designed to separate the active material from STI and the inactive fraction from autolysis products. It was assumed that active and inactive bromoacetone-treated material would behave identically on chromatography at low pH. The separation method could not distinguish between material that had been inactivated by bromoacetone and any other inactive product (*e.g.*, of autolysis) which might have similar chromatographic properties. Such contamination of the inactive bromoacetone-treated material should, however, be low because of the presence of benzamidine in the reaction mixture, which restricts autolysis to a low level.

The properties of active and inactive bromoacetone-treated trypsin were essentially those predicted. While the active material had the same specific activity as purified trypsin, the inactive product contained less than 2% of such activity. The inactive fraction contained 1.06 moles of [^{14}C]acetone/mole in excess of that found in the active material recovered from the same reaction mixture. Thus inactivation appears to require the modification of only one residue. The difference in amino acid composition between active and inactive material of 0.8 residue of histidine unequivocally identified the modified residue. Furthermore, the alkylation product of histidine must have been mainly a monosubstituted product.

The design of the experiment, which prescribed the termination of the reaction when only 50% inactivation had occurred, tended to minimize the accumulation of the products of side reactions proceeding at a slower rate than reaction of histidine. Attempts to obtain complete reaction would have greatly increased the proportion of side-reaction products. The nature of the side reactions is not of importance to the main argument as active trypsin served as internal control. However, it would be of interest to be able to determine where the 1.6 moles of [^{14}C]acetone appearing in the control was located. This could satisfactorily explain the fact that the lysine analyses observed in both active and inactive material are 1.8–2.0 residues lower than the expected composition of trypsin. The side reactions cannot, however, be definitely attributed to lysine without the positive identification of derivatives as it is possible that the disappearance of lysine might be due to a reaction other than alkylation. It may be noted that on analysis of both the active and inactive material, an unidentified product was observed as a small shoulder on the lysine peak.

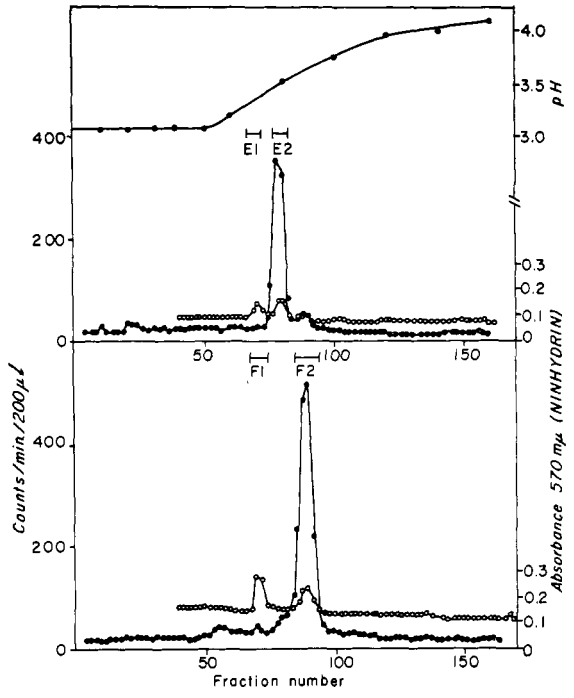

FIGURE 7: Rechromatography of peptides E and F (see Figure 6b) after reduction and carboxymethylation. The same chromatographic system was used as in Figure 6 applying the pH gradient shown at the top of this figure. ○ indicates ninhydrin color. Determinations were not made on early fraction. ● indicates radioactivity. Note that the counts associated with E1 and F1 are probably not significant. Peptides were pooled as indicated.

This product has not yet been identified. It should be possible to identify the exact location of the side chains reacting with bromoacetone from the labeled peptides which have been isolated.

In order to determine the site of inactivation of trypsin with bromoacetone, it was necessary to compare the peptide patterns produced on chromatography of peptic digests of active and inactive material from the same reaction mixture. Although pepsin does not generally show a very high degree of specificity toward protein substrates, it was known that peptides from the histidine loop can be isolated from such a digest (Walsh *et al.*, 1964). The results were unambiguous, however, since four radioactive peaks were observed in both active and inactive material, while two additional peaks appeared in the inactive material only. The four peaks common to both active and inactive material were chromatographically identical in the particular system, suggesting that they contained similar alkylated products, and that the side reactions not involving enzyme inactivation were similar in both cases. Thus the reactivity toward alkylation of side chains is unaffected by inactivation of the enzyme.

The method of purification of the "difference peptides" obtained from inactivated trypsin was based on the supposition that they contained disulfide bonds. Hence they should be cleaved by reduction and additional charged groups could be introduced by subsequent carboxymethyl action. This was indeed found to be the case. The *S*-carboxymethylated peptides were rechro-

matographed, using the same system as was used for the first chromatography, a method first introduced by Spackman *et al.* (1960) to identify the disulfide bonds in ribonuclease and subsequently extended by Brown and Hartley (1966) to paper electrophoresis. The use of the column technique makes it possible, however, to employ a larger quantity of material; moreover, SE Sephadex, in contrast to paper, allowed high recoveries of peptides. It was found that material from each radioactive peak, when treated in this way, gave rise to two peptides, but only one of them contained a significant measure of radioactivity.

The difference peptides isolated evidently originated from the sequence (Walsh *et al.*, 1964):

(29)
Asn-Ser-Gly-Tyr-His-Phe-Cys-Gly-Ser-Leu
|
(Val)-Val-Ser-Ala-Ala-His-Cys-Tyr-Lys-Ser-Gly-
(46) Ile-Gly

The level of [^{14}C]acetone incorporation into the His-46 peptides could account for the observed difference in radioactivity between active and inactive bromoacetone-treated trypsin. These peptides were obtained in a total yield of 49.7% which, considering the low specificity of pepsin and the losses almost invariably involved in peptide purification, suggests that the peptides are representative of the state of alkylation of the trypsin from which they were obtained. The peptide containing histidine-29, which was isolated from both disulfide-containing peptides E and F, contained not more than 7% of the radioactivity of His-46, and this figure is almost certainly an overestimate as no new histidine derivatives could be detected in the histidine-29 peptide. However, the histidine-46 peptides each contained a significant amount of new derivative, or derivatives, emerging from the amino acid analyzer in the position close to that at which tryptophan, had it been present, would have appeared. The acid stability as well as the ninhydrin color factor for this compound, or compounds, is unknown. However, assuming the same factor as for histidine, the yield of this material can be estimated to be 0.73 residue/mole in E2 and 0.75 in F2.

The small quantity of histidine remaining in the histidine-46 peptides (0.2 residue/mole) might arise in part from contamination with some other peptide. However, no other amino acid contaminants were present to the extent of more than 0.1 residue/mole. Another possibility is that a small amount of material inactivated by some other mechanism (*e.g.*, autolysis) might be present. Whatever the origin of this remaining histidine, it represents only a small proportion of the total histidine lost.

It is evident that the inactivation of trypsin by bromoacetone is primarily a result of the alkylation of histidine-46. This residue is much more readily alkylated than is histidine-29, which, under the conditions used here, reacts at most in fractional proportions. The difference in histidine content observed between active and inactive bromoacetone-treated trypsin is sufficiently accounted for by the reaction of histidine-46. It therefore seems unlikely that the third residue, histidine-79, has reacted to any significant degree. This is consistent with the observation that TLCK-trypsin, in which only histidine-46 is alkylated (Shaw and Springhorn, 1967), does not lose histidine when treated with bromoacetone. Bromoacetone is not a bulky reagent and would be expected to find most groups that are not sterically blocked. It contains no charged groups liable to electrostatic interaction with other side chains, nor does it have any apparent structural similarity to a substrate. Thus it would be anticipated that any histidine residues present in unprotonated forms would be at least partially alkylated by bromoacetone. As the inactivation of trypsin occurs at a rate similar to the reaction of bromoacetone with histidine-containing model compounds, it seem probable that the reactivity of histidine-46 in trypsin is similar to that of free histidine. The failure of histidines-29 and -79 to react to a significant extent indicates that they are correspondingly less reactive either for steric or electrostatic reasons, or both. Although His-46 is more reactive than the other two histidines in trypsin, it cannot be said to have unusual reactivity in the sense of the active-center serine.

The observation that histidine-46 is a normal histidine in its reactivity toward halomethyl ketones throws some new light on the reaction of TLCK with the same residue. It seems probable that the selectivity of TLCK results from the high local concentration brought about by the binding affinity of the side-chain groups, rather than from an unusual reactivity of the histidine residue which becomes alkylated.

In attempting to determine which residues in DIP-trypsin had reacted with bromoacetone, essentially similar procedures were employed to those already described. It was, of course, not possible to separate active from inactive material because DIP-trypsin fails to complex with STI (Green, 1953). The analysis of bromoacetone-treated DIP-trypsin indicated the loss of about 0.75 residue of histidine on alkylation. This was in reasonable agreement with the incorporation of [^{14}C]-acetone in the histidine-46 peptides (0.6 residue/mole). Almost all of the [^{14}C]acetone incorporation in the histidine loop peptides was again in the histidine-46 peptide with only minor amounts in the histidine-29 peptide.

Perhaps the most interesting aspect of the reaction of trypsin and its derivatives with bromoacetone is that bromoacetone (in contrast with TLCK) will react with histidine in DIP- (and DPC-) trypsin but that bromoacetone-treated trypsin is no longer reactive with DFP. Thus modification of the active-center serine does not prevent reaction of histidine but modification of histidine apparently leads to loss of the unusual reactivity of serine-183.

Most of the mechanisms which have been proposed for the action of trypsin and its sister enzyme chymotrypsin presuppose that some form of interaction exists between the hydroxyl group of serine and an imidazole nitrogen of histidine to account for the "unusual reactivity" of the serine. The fact that trypsin, after treatment with bromoacetone, becomes inactive toward ester substrates and toward reaction with DFP indicates that

alkylation of histidine-46 destroys the unusual reactivity of the active-center serine, 183. While it might be suggested that loss of the ability to react with DFP could result solely from the steric hindrance imposed on the approach of DFP to the active center by the introduction of an acetonyl group onto histidine, this seems unlikely because of the small size of the group involved. In addition, the fact that histidine-46 can be alkylated in the DIP enzyme at least as rapidly as in the native enzyme indicates the converse, that is, the DIP groups do not hinder the approach of bromoacetone to its site of alkylation. The alkylation of histidine-46 with bromoacetone therefore appears to be the most direct chemical evidence yet obtained for the importance of an interaction between histidine-46 and serine-183 in the catalytic mechanism of trypsin.

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