Methylation of Histidine-57 in α-Chymotrypsin by Methyl *p*-Nitrobenzenesulfonate. A New Approach to Enzyme Modification*

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ABSTRACT: To elucidate further the role of histidine-57 in the mechanism of α-chymotrypsin-catalyzed reactions, the enzyme has been methylated by a nonspecific substrate analog, methyl *p*-nitrobenzenesulfonate. The resulting methylated α-chymotrypsin shows total loss of enzymatic activity toward both the nonspecific substrate, *N-trans*-cinnamoylimidazole, and the specific substrate, *N*-acetyl-L-tryptophan methyl ester. Methyl *p*-nitrobenzenesulfonate is only effective toward α-chymotrypsin and does not inhibit trypsin or subtilisin. Hydrocinnamic acid protects α-chymotrypsin from modification indicating that methylation occurs at the active site. The pH dependency of the rate of inactivation indicates that an ionizing group of $pK = 6.73$, probably histidine, is involved in this methylation. Amino acid analyses prove the loss of one molecule of histidine and the formation of a corresponding amount of 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid (3-methylhistidine). When α-chymotrypsin was modified by [^{14}C]methyl *p*-nitrobenzenesulfonate, the radioactive methyl group was found in the B chain of the enzyme, and high-voltage electrophoresis of the fragments produced by enzymic hydrolysis indicates that mainly histidine-57 was modified. These data provide confirmation that the nitrogen in position 3 of the imidazole ring is involved in the catalytic action of α-chymotrypsin.

Considerable change in enzymic activity upon selective chemical modification of an amino acid residue is one of the most rigorous criteria for showing that this amino acid residue is part of the active center of an enzyme. By using this method, isoleucine-16, histidine-57, methionine-192, and serine-195 have been shown to be present at or near the active site of α-chymotrypsin (Koshland *et al.*, 1962; Cunningham, 1965; Bender and Kézdy, 1965; Bruice and Benkovic, 1966).

Several studies have been reported indicating the presence of histidine-57 at the active center of α-chymotrypsin. Photooxidation was used by Weil and coworkers (1953) and by Koshland *et al.* (1960, 1962). Schoellmann and Shaw (1963) reported that TPCK[1] acts as a modifying reagent for histidine-57. Stevenson and Smillie (1965) reported that phenoxymethyl chloromethyl ketone modified histidine-57 and Schramm (1965) reacted histidine-57 with 2-phenyl-1,4-dibromoacetoin. However, these chemical modifications of histidine-57 do not rigorously prove the catalytic function of the histidine residue in chymotrypsin, since the alkylating reagents are specific substrate analogs: after reacting with histidine these reagents occupy the active site and thus could cause the loss of enzyme activity by steric inhibition. In the case of photooxidation, the reaction is far from being specific toward histidine: the loss of tryptophan (Weil *et al.*, 1953) or methionine (Koshland *et al.*, 1962; Ray and Koshland, 1960) was also observed.

Physico-chemical approaches on the α-chymotrypsin reaction mechanism, such as the determination of the pH dependency of the enzyme reaction and thermodynamic studies of ionizing groups provide only indirect evidence that histidine is involved in the catalytic action. The amino acid sequences of chymotrypsin, trypsin, and elastase show great similarities near the histidine residue. These data indicate again the importance of the histidine residue but do not specify its catalytic role.

The uncertainties concerning the mechanistic participation of histidine can be overcome if the modifying group is small. An ideal reagent would then be composed of two elements: a small modifying group attached to a large, hydrophobic molecule which will bind to the active site. After modification of the latter by the small group, the carrier group would be released. For this purpose, a nonspecific substrate analog, methyl *p*-nitrobenzenesulfonate, was synthesized and used to methylate α-chymotrypsin, since an alkyl group of a sulfonic acid ester is a good leaving group (Hine, 1962). Also the methyl group is small, it cannot fill up the "hydrophobic pocket" and—as Koshland pointed out—it would not disturb the steric properties of α-chymotrypsin (Koshland *et al.*, 1962).

In this paper the methylation of the histidyl residue in α-chymotrypsin by methyl *p*-nitrobenzenesulfonate is reported. Furthermore, we will attempt to define further the function of histidine in α-chymotrypsin-catalyzed reactions by studying the kinetic behavior of the modified enzyme.

During the modification study, it became necessary to remove some unknown impurities from commercial chymotrypsin and a purification method using CM-Sephadex was developed. This purification method is also described in this paper.

Experimental Section

Materials and Methods. CHEMICALS. *p*-Nitrobenzenesulfonyl chloride and hydrocinnamic acid were purchased from East-

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[1] Abbreviation used is: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

man Kodak. The former compound was recrystallized from hexane before use [mp 77–78°, lit. mp 80° (Bell, 1928)]. *N*-Acetyl-L-tryptophan methyl ester (H. M. Chemical Co.) was recrystallized once from ethyl acetate [mp 154–155°, lit. mp 152–155° (Huang and Niemann, 1951)]. *N-trans*-Cinnamoylimidazole and imidazole [mp 89°, lit. mp 90–91° (Hofmann, 1953)] were products of Aldrich Chemicals. *N-trans*-Cinnamoylimidazole was recrystallized from dry hexane [mp 134°, lit. mp 133–133.5° (Schonbaum *et al.*, 1961)]. *N*-Acetyl-L-serine amide [mp 137–138°, lit. mp 138–139° (Rothstein, 1949)], *N*-acetyl-L-methionine amide [mp 124–125°), L-isoleucine amide hydrochloride, and *p*-nitrophenyl-N^2-benzyloxycarbonyl-L-lysinate hydrochloride were obtained from Cyclo Chemical Co. The latter compound was recrystallized from acetonitrile–ethanol mixture [mp 150–152°, lit. mp 150–151° (Bender *et al.*, 1966a)]. Acetonitrile (Nano grade) was purchased from Mallinckrodt Chemical Works. Urea (Baker Chemical Co.) was recrystallized twice from water [mp 133–135°, lit. mp 132.7° (Lange, 1956)]. Sephadex G-25 and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemical, Inc. DEAE-cellulose (0.87 mequiv/g), and 2-amino-3-(1-methyl-5-imidazolyl)- and 2-amino-3-(1-methyl-4-imidazolyl)propanoic acids, were obtained from Calbiochem. Tris(hydroxymethyl)aminomethane was a primary standard (Sigma Chemical Co.). [^{14}C]Methanol (2 mCi/mmole) and Omnifluor were purchased from New England Nuclear Corp. The other chemicals were reagent grade and were used without further purification.

Methyl *p*-nitrobenzenesulfonate was synthesized according to the method of Morgan and Cretcher (1948). The product was recrystallized from ligroin (mp 91.5–92.5°). The purity of the methyl ester was examined by thin-layer chromatography with benzene–chloroform mixture (1:1, v/v). A trace amount of the starting compound was detected. The infrared absorption spectra by Beckman IR-10 with KBr pellet showed strong absorptions at 1540, 1370, 1360, 1190, 980, and 790 cm^{-1}. The ultraviolet absorption in 0.1 M sodium phosphate buffer (pH 7.84) showed a λ_{max} of 253 mμ and an $\epsilon_{253\,m\mu}$ of 13.1 $\times 10^3$(*p*-nitrobenzenesulfonic acid showed its absorption maximum at 265 mμ, with an $\epsilon_{265\,m\mu}$ of 8.3 $\times 10^3$). The nuclear magnetic resonance (Varian A-60) absorption bands of the methyl ester were at 8.66 ppm (quartet) and at 4.06 ppm (singlet), and the relative area ratio was 4:3. *Anal.*[2] Calcd for $C_7H_7NO_5S$: C, 38.7; H, 3.2; N, 6.4; S, 14.7. Found: C, 39.3; H, 3.4; N, 6.6; S, 14.4. The solubility of this ester is 4.8×10^{-3} M in a 0.1 M sodium phosphate buffer solution containing 12.5% acetonitrile.

Radioactive methyl ester was prepared from [^{14}C]methanol. Radioactivity was determined by a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.) in a water miscible scintillation solution (Bray, 1960). The efficiency was 65.9% by using [^{14}C]toluene as an internal standard.

Enzymes. α-Chymotrypsin (EC 3.4.4.5, salt free, three-times recrystallized) was purchased from Worthington. Porcine trypsin (EC 3.4.4.4) was the product of Armour Pharmaceutical Co., and subtilisin (Subtilopeptidase A, EC 3.4.4.16) was obtained from Novo Industry, Denmark. Pepsin (EC 3.4.4.1, hog stomach, three-times recrystallized) was obtained from Mann Research Laboratories.

The active α-chymotrypsin concentration was determined by titration with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). The rate assays of native and modified enzymes were carried out by using *N*-acetyl-L-tryptophan methyl ester as a substrate (Zerner *et al.*, 1964). The activities of trypsin and subtilisin were determined by a "burst" titration method (Bender *et al.*, 1966a).

All assays were carried out with Cary spectrophotometers, Model 14. The spectrophotometers were placed in a constant-temperature room (25°). Sample and reference compartments were kept at 25 ± 0.5° with a constant-temperature water circulator. Quartz cuvets (3 ml) with a 1.0-cm light path were used.

CM-Sephadex C-50 was used for the purification of commercial α-chymotrypsin. CM-Sephadex was equilibrated with 0.01 M sodium citrate buffer (pH 5.5), and was packed by gravity flow in a chromatographic tube (2 × 20 cm). The enzyme was eluted from the column with a linear sodium chloride gradient (0.01 M sodium citrate buffer–1 M sodium chloride in 0.01 M sodium citrate buffer, pH 5.5). The column shrank with increasing salt concentration and the height became half the original value at the end of the separation. However, the flow rate (25 ml/hr) did not change noticeably.

The eluted enzyme fractions were combined. The enzyme solution was cooled in an ice bath while powdered ammonium sulfate was added to make a 0.7 saturated solution. The required amount of ammonium sulfate was calculated according to Noltmann *et al.* (1961). After stirring for 30 min, the enzyme was collected by centrifugation (Servall SS-34 rotor) at 8000 rpm, 0° for 20 min. The enzyme was redissolved in water and dialyzed against water or an appropriate buffer solution for 6 hr. All purification procedures were carried out in a cold room at 4°.

The pH values of all solutions were determined by a Radiometer pH meter type PHM 4c with a type B electrode.

Modification of α-Chymotrypsin by Methyl p-Nitrobenzenesulfonate. α-Chymotrypsin was dissolved in an appropriate buffer solution and the concentration was adjusted to $3 \sim 7 \times 10^{-4}$ M. Methyl *p*-nitrobenzenesulfonate in acetonitrile (~0.04 M) was added in excess to the enzyme solution. For analytical purposes, the enzyme (~500 mg) was dissolved in a sodium phosphate buffer (pH 7.9). Since the half-life of the ester in aqueous solution is about 60 min, the ester solution was added three times (in excess of the enzyme concentration) during the 6-hr incubation period. After the modification was complete, the enzyme solution was dialyzed against double-distilled water in a cold room (4°). The water was changed three times in the 12-hr dialyzing period. [^{14}C]Methylchymotrypsin was prepared by using [^{14}C]methyl *p*-nitrobenzenesulfonate (specific activity 21.9×10^{10} cpm/mole) in acetonitrile in the same manner as described above.

Modifications of trypsin and subtilisin were attempted under the same conditions as those used in the modification of α-chymotrypsin.

Separation of B and C Chains of α-Chymotrypsin. The disulfide bonds in chymotrypsin were cleaved by applying the method developed by Pechère *et al.* (1958) to cleave chymotrypsinogen. DEAE-cellulose was washed as described by Peterson and Sober (1962) and equilibrated with a sodium phosphate buffer containing 8 M urea. The column (3.5 × 25 cm) was packed under slight pressure. The C and B chains were eluted with 500 ml of 0.02 M sodium phosphate (pH 7.91)–8 M urea, and with 500 ml of 0.06 M sodium phosphate

[2] Analysis by Micro-Tech Laboratories, Inc., Skokie, Ill.

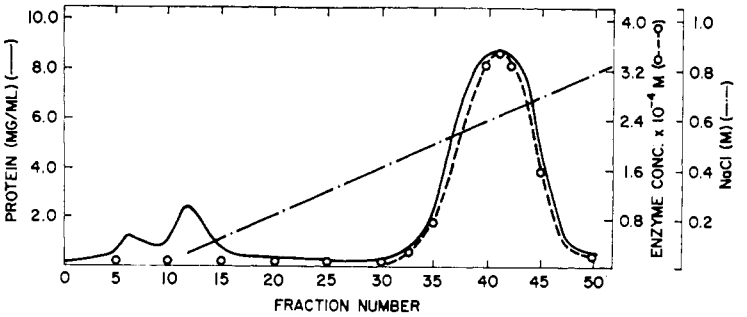

FIGURE 1: Purification of α-chymotrypsin by CM-Sephadex. Commercial α-chymotrypsin was purified on CM-Sephadex C-50 (2 × 20 cm). The enzyme was eluted from the column with a 0.01 M sodium citrate–1 M NaCl gradient (pH 5.5) at 4°. The flow rate was 25 ml/hr. Each 5-ml fraction was collected in a tube. The absorption at 280 mμ was measured by a Gilson ultraviolet absorption apparatus which was connected to Beckman fraction collector and to a Texas Instrument recorder. The enzyme activity was determined by *N-trans*-cinnamoylimidazole titration.

(pH 7.89)–8 M urea, respectively. Eluent (10 ml) was collected in each test tube with a flow rate of 60 ml/hr. The protein concentration of the eluent in each tube was determined by its absorbance at 280 mμ.

Electrophoresis. Electrophoreses of the enzymes on cellulose acetate sheets (2.5 × 19 cm) were carried out in 0.1 M Tris-HCl buffer (pH 8.66), at 14 V/cm for 2 hr, with a Gelman horizontal electrophoresis unit. Electropherograms were obtained by staining with Ponceau S in 3% trichloroacetic acid. The electropherograms were fixed in 5% acetic acid.

High-voltage paper electrophoresis was carried out with a Savant Flat or Tank apparatus, according to Brown and Hartley's description (1966). Peptides and amino acids were detected by fluorescence under ultraviolet light and also by spraying with ninhydrin and Pauly reagent (Smith, 1960). When the radioactive ester was used for the modification, radioautograms were prepared by using X-ray film (Du Pont Cronex III, 35.5 × 43 cm).

Paper Chromatography. Ascending paper chromatography (Whatman No. 1) was carried out in a solvent system of 1-butanol–acetic acid–water (6:4:1, v/v).

Amino Acid Analysis. Protein (native and modified chymotrypsins, B and C chains) was hydrolyzed for 20 hr in 6 N HCl at 110°. Amino acid analyses were performed by using Spinco amino acid analyzers Model 120 B and 120 C with regular Spinco AA-15 and AA-28 columns. Beckman PA-35 column for physiological fluid analyses was also used in the basic amino acid determinations.

Results

Purification of Commercial α-Chymotrypsin. The preliminary experiments showed that the inhibition of the esterase activity of commercial α-chymotrypsin did not obey first-order kinetics, similar to the case of the inhibition by TPCK (Kézdy *et al.*, 1967). Purification of the enzyme by gel filtration on Sephadex G-25 (Yapel *et al.*, 1966) still did not produce satisfactory kinetics. Moreover, cellulose acetate electrophoreses of both commercial enzymes and those purified by Sephadex G-25 revealed two extra proteins. Thus it was necessary to purify further the commercial enzyme in order to eliminate these contaminants. The elution pattern from CM-Sephadex C-50 column is shown in Figure 1.

The main fractions were combined and the protein was precipitated at 0.7 saturation of ammonium sulfate. This method was preferred to lyophilization because the latter method produces some water-insoluble material.

By titration, commercial chymotrypsin usually shows a purity of 80% (assuming mol wt 24,800), which increases to nearly 100% after purification by CM-Sephadex column chromatography. The purified enzyme preparation has a λ_{max} of 281 mμ, and an $\epsilon_{281\ m\mu}$ of 52.05×10^3 Δμ/mole per l. per cm.

Inhibition of α-Chymotrypsin by Methyl p-Nitrobenzenesulfonate. To examine the inhibitory action of the methyl ester on α-chymotrypsin, the loss of the chymotryptic activity was measured by two assay methods, *N-trans*-cinnamoylimidazole (nonspecific substrate) titration and the rate assay method with *N*-acetyl-L-tryptophan methyl ester (specific substrate).

The percentage of remaining activity was plotted against the incubation time, as shown in Figure 2. The inhibitory action of *p*-nitrobenzenesulfonic acid was examined under the same conditions with the latter compound. α-Chymotrypsin showed only a 0.7% loss of activity after 30 min and a 7.4% loss after 1 hr. No inhibitory effect of acetonitrile (9.9%, v/v) was observed during these incubation periods. The slightly higher residual activity found by the rate assay method was probably caused by the dilution of the enzyme. The loss of the enzymatic activity determined by both assay methods was actually almost identical.

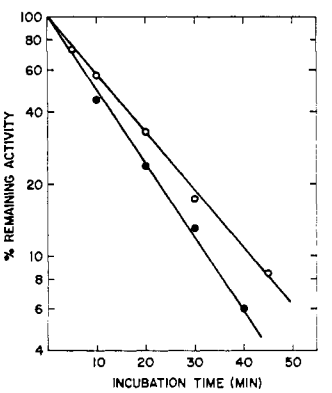

FIGURE 2: Loss of α-chymotrypsin activity upon incubation with methyl *p*-nitrobenzenesulfonate. Purified α-chymotrypsin in 0.1 M sodium phosphate buffer, pH 7.93 (3.65×10^{-4} M), was incubated with methyl *p*-nitrobenzenesulfonate (2.86×10^{-3} M) at 25°. The concentration of CH_3CN was 9.9%. The remaining activity was measured by *N-trans*-cinnamoylimidazole titration (full circles) and by a rate assay method with *N*-acetyl-L-tryptophan methyl ester (2.48×10^{-4} M, open circles).

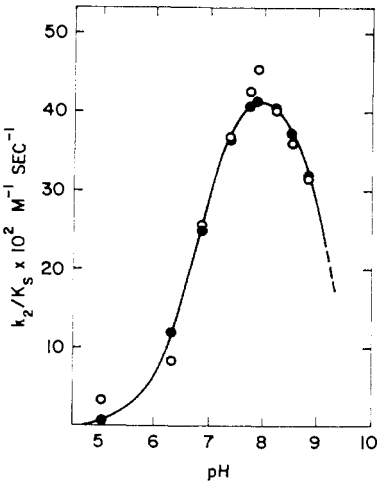

FIGURE 3: pH–inhibition profile. The data in Table II are plotted on a graph. The curve is the theoretical bell-shaped curve (full circles) described by the equation:

$$k_2/K_s = 47 \times 10^{-2}/(1 + [H]/10^{-6.78} + 10^{-9.14}/[H])$$

The experimental value, $k_{exp}/[I_0]$, is shown by open circles.

When trypsin and subtilisin activities were tested under identical conditions no loss of activity was observed.

Protective Effect of Hydrocinnamic Acid against the Inhibitor. Hydrocinnamic acid is a competitive inhibitor of α-chymotrypsin (Kaufman and Neurath, 1949). If methyl *p*-nitrobenzenesulfonate is reacting at the active site then hydrocinnamic acid should be a competitive inhibitor of this process. The results (Table I) show that hydrocinnamic acid definitely decreases the inhibitory action of the methyl ester by protecting the active site of α-chymotrypsin.

pH Dependence of Inhibition of α-Chymotrypsin by Methyl p-Nitrobenzenesulfonate. Preliminary experiments indicated that in order to achieve a high reaction rate for the modification of α-chymotrypsin, the pH must be 7.2 or higher. This observation already suggested that the modification might involve a histidyl group.

TABLE I: Protection from Modification of α-Chymotrypsin by Methyl *p*-Nitrobenzenesulfonate Using Hydrocinnamic Acid.[a]

Concn of the Methyl Ester $\times 10^4$ M	Concn of Hydrocinnamic Acid $\times 10^4$ M	Act. Loss (%)
6.67	0	60.8
6.67	6.67	36.3

[a] α-Chymotrypsin solution in 0.1 M sodium phosphate, pH 7.84 (enzyme concentration 3.86×10^{-4} M), was incubated as shown in the table for 6 hr at 25°. The concentration of acetonitrile was adjusted to 12.5% in the final incubation mixture. After 6-hr incubation, the mixture was dialyzed for 12 hr with three changes in a cold room (4°). The activity was determined by *N-trans*-cinnamoylimidazole titration.

The pH profile of the inhibition of chymotrypsin activity by the methyl ester was measured in some detail. The loss of enzymatic activity at each pH was measured in a tenfold excess of the methyl ester with respect to the enzyme concentration (~4×10^{-4} M). The pH dependence of the second-order rate constant calculated by dividing the experimental first-order rate constant by the inhibitor concentration is shown in Table II and Figure 3. The pH – k_{inact} was a bell-shaped curve, similar to pH dependency of the modification of chymotrypsin by TPCK (Schoellman and Shaw, 1963; Kézdy *et al.*, 1967). The pH values were calculated according to a graphical method (Bender *et al.*, 1964). We obtained the following values: $pK_1 = 6.73$ and $pK_2 = 9.14$. The low rate of inhibition at higher pH (above pH 8) can be visualized as an intramolecular competitive inhibition by the N-terminal isoleucine (Bender *et al.*, 1966b; Kézdy *et al.*, 1967; Sigler *et al.*, 1968).

Effect of the Inhibitor Concentration on the Enzyme Concentration. If the methyl ester is bound to the enzyme before irreversibly inhibiting the enzymatic activity by methylation, then the following scheme holds:

$$[E] + [I] \overset{K_s}{\rightleftharpoons} [EI] \xrightarrow{k_2} [E^*] \text{ (inactive)} + P$$

where $[E]$ is the concentration of the free enzyme, $[EI]$ the concentration of the enzyme–inhibitor complex, $[E^*]$ the methylated enzyme, and P the nitrobenzenesulfonate ion.

Under the condition of $[E_0] \gg K_s$, the reaction should obey second-order kinetics, therefore

$$\frac{k_2}{K_s} t = \frac{1}{[E_0] - [I_0]} \ln \frac{([E_0] - [E^*])[I_0]}{([I_0] - [E^*])[E_0]}$$

$[E_0]$ and $[E^*]$ are experimentally measurable and $[I_0]$ is known. From this equation, k_2/K_s must be constant under different ratios of $[E_0]$ to $[I_0]$. Experiments were carried out at pH 7.85 for 1-hr incubation periods with various inhibitor concentrations and k_2/K_s was calculated according to the equation above. The results are shown in Table III.

As seen in Table III, k_2/K_s is constant within experimental error at different initial inhibitor concentrations.

Effect of the Functional Amino Acid Side Chains of the Active Center of α-Chymotrypsin on the Spontaneous Hydrolysis of Methyl p-Nitrobenzenesulfonate. Since methyl *p*-nitrobenzenesulfonate seems to bind to the active site of α-chymotrypsin before methylating the enzyme, it was interesting to see whether amino acid residues at the active site would react with the ester in solution. For this reason the spontaneous hydrolysis rate of methyl *p*-nitrobenzenesulfonate has been determined in the presence of the proposed functional amino acids of α-chymotrypsin. The results are shown in Table IV.

The inactivation rate of chymotrypsin by the methyl ester is at least 200 times faster than the nonenzymic rate of reaction of the methyl ester with imidazole at the concentration of 5.00 $\times 10^{-2}$ M. Histidine in 0.1 M sodium phosphate buffer (pH 7.94) was incubated with an excess of methyl ester for 48 hr. The majority of the histidine was unattacked but a small amount of 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid (1-methylhistidine) and a trace amount of 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid (3-methylhistidine) were detected by amino acid analysis.

TABLE II: pH – k_{inact} Profile of α-Chymotrypsin by Methyl *p*-Nitrobenzenesulfonate.[a]

pH	Buffer (0.1 M)	$[I_0] \times 10^3$ M	$k_{exp} \times 10^4$ sec^{-1}	$k_{exp}/[I_0] \times 10^2$ M^{-1} sec^{-1}	$k_2/K_s \times 10^2$[b] M^{-1} sec^{-1}
5.01	Na acetate	3.85	1.3 ± 0.18	3.3	0.78
6.32	Na phosphate	3.85	3.2 ± 0.48	8.3	12.10
6.84	Na phosphate	4.55	11.6 ± 1.10	25.5	25.05
7.36	Na phosphate	4.54	16.7 ± 1.41	36.8	36.73
7.74	Na phosphate	4.54	19.3 ± 1.38	42.5	40.83
7.92	Na phosphate	4.54	20.6 ± 0.97	45.4	41.49
8.22	Tris-HCl	4.54	18.3 ± 1.33	40.3	40.64
8.52	Tris-HCl	4.54	16.4 ± 1.41	36.1	37.36
8.82	Tris-HCl	4.76	15.0 ± 1.51	31.5	31.59

[a] α-Chymotrypsin was incubated with a tenfold excess of the methyl ester at various pH values. At least five values of remaining activity were determined by *N-trans*-cinnamoylimidazole titration at each pH and from these data pseudo-first-order rate constants (k_{exp}) were calculated. [b] Calculated with the equation $k_2/K_s = 47 \times 10^{-2}/(1 + [H]/10^{-6.78} + 10^{-9.14}/[H])$.

These facts indicate that the methyl ester must be oriented in the right direction on the surface of the enzyme, and that the enzyme truly catalyzes the reaction of the ester with histidine. The importance of the enzymatic catalysis is also emphasized by the fact that 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid is the primary product by a chemical reaction. However, 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid is the only product by the modification as described in the latter section.

Spectrophotometric Properties of Native and Modified Chymotrypsin. If *p*-nitrobenzenesulfonate (λ_{max} 265 mμ) had been covalently attached to enzyme, sizable spectroscopic changes should be observed in an 8 M urea solution (Gold and Fahrney, 1964). Our results indicate that no sulfonylation occurs at serine-195 (Figure 4).

The difference spectra of modified enzyme *vs.* native enzyme showed small negative absorptions at 285 and 292 mμ as shown in Figure 4. These spectra are comparable with the difference spectra of chymotrypsin caused by substrates (Benmouyal and Trowbridge 1966; Himoe *et al.*, 1967). Thus the perturbation of the tyrosyl group by methylation of the active site is probably similar to the perturbation caused by enzyme–substrate complex formation or acylation.

Identification of the Methylated Site on the Modified Enzyme. *S*-Sulfochymotrypsin was prepared and the B and C chains were purified using the method that Pechère *et al.* (1958) used to purify chymotrypsinogen. The B and C chains of native

TABLE III: The Relationship between $[I_0]/[E_0]$ and k_2/K_s.[a]

$[I_0] \times 10^4$ M	$[I_0]/[E_0]$	$k_2/K_s \times 10^2$ M^{-1} sec^{-1}
0.61	0.21	33.6
2.13	0.72	36.3
3.03	1.03	38.9
3.61	1.24	34.6
5.15	1.74	33.0
7.60	2.58	29.6
8.30	2.82	33.9
10.65	3.60	28.5

[a] α-Chymotrypsin was dissolved in 0.1 M sodium phosphate buffer, pH 7.85 (2.96×10^{-4} M), and the acetonitrile concentration adjusted to 9% in the final mixture after the required amount of the methyl ester in acetonitrile was added. After incubation for 1 hr at 25°, the remaining α-chymotrypsin concentration was determined by *N-trans*-cinnamoylimidazole titration.

TABLE IV: The Effect of the Functional Amino Groups on the Spontaneous Hydrolysis of Methyl *p*-Nitrobenzenesulfonate.[a]

Methyl *p*-Nitrobenzenesulfonate Concn × 10^5 M	Amino Acid	Concn (M)	$k_{exp} \times 10^4$ sec^{-1}
4.17			1.77
5.16	Isoleucinamide	8.01×10^{-4}	1.61
4.14	*N*-Acetyl-L-methioninamide	5.44×10^{-4}	1.73
4.14	*N*-Acetyl-L-serinamide	1.74×10^{-3}	1.67
4.01	Imidazole	7.26×10^{-4}	1.55
4.01	Imidazole	5.00×10^{-2}	2.88
($k_2/K_s = 2.2 \times 10^{-3}$ M^{-1} sec^{-1})			
(Inhibition of chymotrypsin $k_{exp}/[I_0] = 45.4 \times 10^{-2}$ M^{-1} sec^{-1} from Table II)			

[a] The buffer used was 0.1 M sodium phosphate, pH 7.94. Methyl *p*-nitrobenzenesulfonate was dissolved in acetonitrile. The amino acid derivatives were in aqueous solutions. The decomposition of the ester was measured at 253 mμ at 25°.

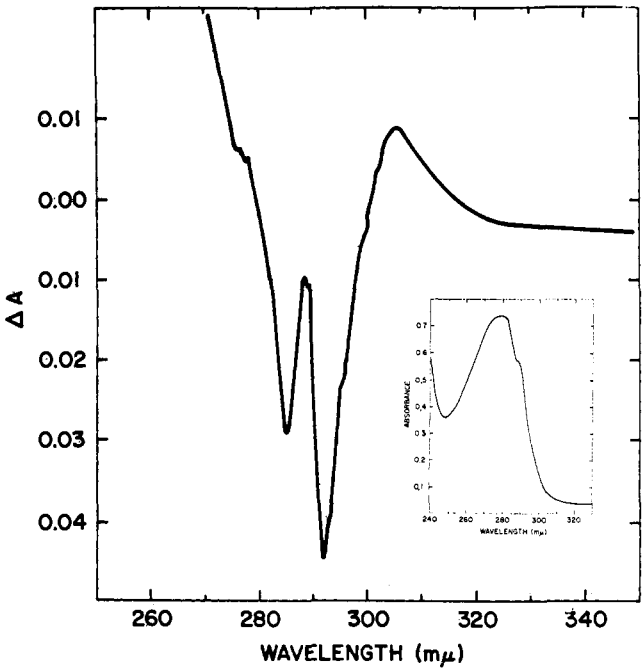

FIGURE 4: Spectrophotometric properties of modified α-chymotrypsin. Both native and modified chymotrypsins were dissolved in (pH 9.41) buffer solution and adjusted to an absorbance of 0.546 at 281 mμ. The native enzyme solution was placed in the reference compartment and the modified one was put in the sample compartment of a Cary spectrophotometer. The inserted figure shows the ultraviolet absorption of modified enzyme in an 8 M urea aqueous solution *vs.* an 8 M urea solution.

chymotrypsin were separated by stepwise elution (Figure 5) instead of gradient elution from a DEAE-cellulose column.

After α-chymotrypsin (522 mg in 10 ml of sodium phosphate buffer, pH 7.8, nitrogen 1.84 mg/ml) was modified by [^{14}C]-methyl *p*-nitrobenzenesulfonate, the resulting [^{14}C]methylchymotrypsin had a total radioactivity of 4.5 × 10^6 cpm (5.7 × 10^4 cpm/mg of N), after removal of the reagents by dialysis. Calculations showed that the binding of 1 mole of the reagent would result in a labeled chymotrypsin with a total radioactivity of 4.6 × 10^6 cpm. Thus chymotrypsin reacted almost stoichiometrically with the [^{14}C]methyl group. After the disulfide linkages of the modified enzyme were cleaved to the *S*-sulfonate, modified *S*-sulfochymotrypsin was chromatographed to separate the B and C chains. The radioactivity in each tenth tube was measured by a liquid scintillation counter. The elution patterns of protein and radioactivity are shown in Figure 6. The main protein fractions of B and C chains were dialyzed against 6 l. of 0.01 M acetic acid for 12 hr with three changes. Aggregated proteins were centrifuged (Servall SS-34 rotor) at 8000 rpm for 20 min, and the precipitated proteins of B and C chains were dissolved in 0.05 M sodium phosphate, pH 7.8. The solution of B chain showed a radioactivity of 3.4 × 10^4 cpm/mg of N and a radioactivity of the C chain solution was 0.5 × 10^4 cpm/mg of N. The loss of radioactivity was probably caused by the loss of protein during the series of dialysis (according to Craig (1960) a 50% escape of α-chymotrypsin in 0.01 N acetic acid occurs in 5 hr with a 20/32 dialysis membrane). The main radioactivity peak corresponded to the main protein peak of the B chain as measured by ultraviolet absorption.

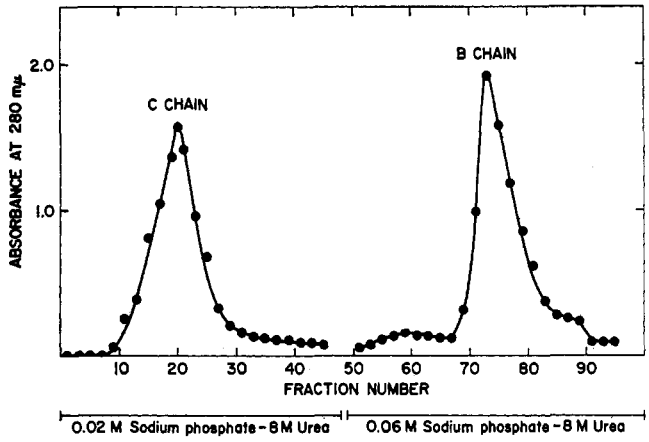

FIGURE 5: Separation of B and C chains of native chymotrypsin. Chains B and C were separated on a DEAE-cellulose column (3.5 × 25 cm) by stepwise elution with 0.02 M sodium phosphate (pH 7.91)–8 M urea, and 0.06 M sodium phosphate (pH 7.89)–8 M urea. The 10-ml fractions were collected in tubes and the flow rate was 60 ml/hr.

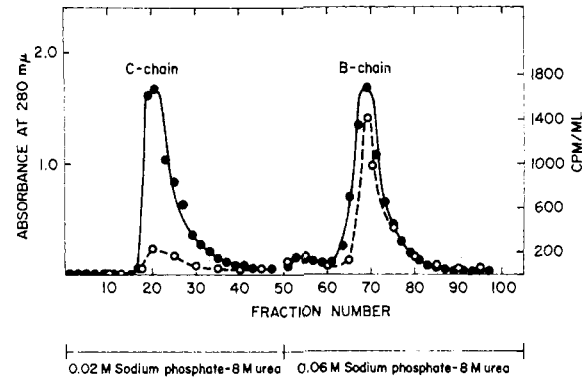

FIGURE 6: Separation of B and C chains of [^{14}C]methyl-modified chymotrypsin. The separation method was the same as that for native chymotrypsin. An aliquot of 250 μl of eluent of each tenth tube was used for the radioactivity measurement; (●—●) protein, (○—○) radioactivity.

High-voltage electrophoresis was applied to the peptic digestion of native and modified chymotrypsins and of their B chains. A pyridine–acetic acid–water buffer (pH 6.5) as reported by Brown and Hartley (1966) was used. The spots corresponding to histidine-57 were very faint in the modified B chain and modified chymotrypsin after spraying with Pauly reagent.

The radioautograph of B chain showed high intensity of radioactivity at the spot of histidine-57 and low intensity at the spot of histidine-40, with no radioactivity observed in other areas. The radioautograph of modified chymotrypsin (Figure 7) revealed very weak radioactivity in an extra Pauly reagent positive spot (corresponding to 1D2 by Brown and Hartley; probably tyrosine-171) after the film was exposed more than 3 weeks.

Amino acid analyses of native and modified chymotrypsins were identical within experimental error except for the disappearance of one molecule of histidine and the appearance of one molecule of 2-amino-3-(1-methyl-4-imidazolyl)propanoic

TABLE V: Amino Acid Compositions of α-Chymotrypsin and α-Chymotrypsin Modified by Methyl *p*-Nitrobenzenesulfonate.

Amino Acid[a]	α-Chymotrypsin	Modified α-Chymotrypsin	Reference[b]
Aspartic acid	22.0	21.9	21.4
Threonine[c]	21.3	21.0	21.8
Serine[c]	25.5	23.5	27.2
Glutamic acid	14.8	14.2	15.3
Proline	9.3	10.0	9.0
Glycine	22.7	24.6	23.4
Alanine	22.1	22.6	22.5
Valine	20.3	20.7	21.9
Methionine	1.9	1.8	2.1
Isoleucine	7.5	6.9	9.9
Leucine	18.2	17.2	19.0
Tyrosine[c]	3.6	3.1	4.1
Phenylalanine	5.5	5.6	6.1
Lysine[d]	13.5	13.0	13.4
Histidine[d]	2.0	0.8	1.7
3-Methylhistidine[d]		1.1	
Arginine	2.6	3.0	2.7

[a] Hydrolysis was carried out for 20 hr at 110°. [b] Data were obtained from Lawson and Schramm (1965). [c] Numbers were uncorrected for recovery. [d] Data were obtained by using a PA-35 column.

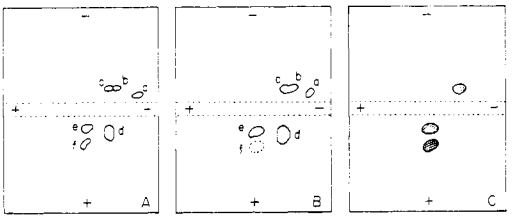

FIGURE 7: Diagonal high-voltage electropherogram. Protein was digested by pepsin in 0.005 N HCl for 12 hr at 36° and applied to Whatman No. 3MM paper (30 × 58 cm). Two-dimensional electrophoresis was carried out in pyridine buffer (pH 6.5) at 60 V/cm for 2 hr. Oxidation of chromatogram was carried out in performic acid fumes for 2 hr using the method of Brown and Hartley (1966). After spraying the chromatogram with Pauly reagent, a radioautogram was prepared by using X-ray film (Du Pont Cronex III, 35.5 × 43 cm). The film was exposed for 3 weeks and then was developed: (A) native chymotrypsin, (a) pink, (b) orange, (c) pink-orange, (d) pink-orange, (e) pink, (f) pink; (B) modified chymotrypsin by the radioactive ester (a) pink, (b) orange, (c) orange, (d) pink-orange, (e) pink-orange, (f) faint pink; (C) distribution of radioactivity on the radioautogram.

acid per enzyme molecule. The amino acid compositions of native and modified α-chymotrypsin are shown in Table V. The B and C chains were also subjected to amino acid analysis. These results are shown in Table VI.

Column chromatography (Ong *et al.*, 1965; Tobita and Folk, 1967), high-voltage electrophoresis (Brown and Hartley, 1966), and paper chromatography were applied to purify the histidine-40 and histidine-57 peptides. Both peptides were contaminated by other minor peptides. Amino acid analyses indicated that the two peptides were indeed the histidine-40- and histidine-57-containing peptides, but the data were not as accurate as we hoped for.

As a conclusion these results indicate that methylation occurred mainly at histidine-57 of α-chymotrypsin and 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid was the product.

Discussion

The modification of α-chymotrypsin by methyl *p*-nitrobenzenesulfonate has some unique features compared with the other enzyme modifying reagents which have been summarized by Singer (1967) and Baker (1967). Several reagents for modifying chymotrypsin have been reported; those which react with histidine include TPCK (Schoellmann and Shaw, 1963), phenoxymethyl chloromethyl ketone (Stevenson and Smillie, 1965), and 2-phenyl-1,4-dibromoacetoin (Schramm, 1965). These known histidine-modifying reagents have a common structural feature: they are more or less close analogs of derivatives of aromatic amino acids, for which α-chymotrypsin shows a high specificity. In contrast, methyl *p*-nitrobenzenesulfonate has a structure analogous to nonspecific substrates, such as esters of benzoic acid.

This reagent modifies histidine in α-chymotrypsin and inactivates the enzyme. It is reacting only with α-chymotrypsin and it does not affect the activity of trypsin and subtilisin—other serine proteinases.

Ryle (1966) and Singer (1967) pointed out that when an active site of an enzyme is labeled, and the enzyme loses its activity, the results must be examined critically, since the loss of activity does not necessarily mean that the active center has been modified chemically.

When it was found that methyl *p*-nitrobenzenesulfonate inhibited α-chymotrypsin, attention was focused on what amino acid residue in the enzyme was modified, and whether methylation or sulfonylation of this residue had taken place.

The modified enzyme showed an ultraviolet absorption spectrum almost identical with that of the native enzyme with a λ_{max} of 281 mμ. The difference spectrum of the modified chymotrypsin against the native enzyme showed negative absorptions at 285 and 292 mμ, and a positive absorption at 288 mμ. Such spectral changes have been observed when α-chymotrypsin formed a complex with *N*-acetylphenylalanine ethyl ester (Benmouyal and Trowbridge, 1966) or by monoacetylchymotrypsin formation with *o*-nitrophenyl acetate (Himoe *et al.*, 1967). These changes were interpreted to be the result of the perturbation of the tyrosyl groups by the enzyme-specific substrate complex or serine acyl group.

The pH – k_{inact} profile showed that an ionizing group with $pK_1 = 6.73$ was involved in the modification, probably histidine. Also hydrocinnamic acid, a competitive inhibitor of α-chymotrypsin, showed a protective action against the methyl ester. These facts indicated that the modification occurred at the active site.

The following results support the conclusion that α-chymotrypsin is methylated rather than sulfonylated by the methyl sulfonate. An ultraviolet spectral change was not observed after the enzyme was modified as described above. Also a spectral shift was not observed when the modified enzyme was

TABLE VI: Amino Acid Compositions of Chains B and C before and after Modification of Chymotrypsin with Methyl *p*-Nitrobenzenesulfonate.

	B Chain[a]			C Chain[b]		
	From the Sequence[c]	Before	After	From the Sequence[c]	Before	After
Aspartic acid	13	13.0	13.0	9	9.0	9.0
Threonine[d]	13	11.6	13.2	9	10.1	9.9
Serine[d]	15	16.4	15.6	11	9.6	9.7
Glutamic acid	9	9.5	8.6	4	3.7	3.3
Proline	3	3.7	3.7	4	5.4	5.7
Glycine	12	11.7	11.8	10	12.1	10.7
Alanine	11	10.6	10.5	10	10.5	10.5
Valine[d]	13	11.4	11.4	8	6.4	7.0
Methionine	0	Trace	0.01	2	1.9	1.8
Isoleucine	6	6.0	5.5	3	3.1	2.1
Leucine	9	10.3	9.5	8	8.7	7.8
Tyrosine[d]	2	1.3	1.3	2	2.4	1.2
Phenylalanine	6	5.5	5.7	0	0	0
Lysine[e]	8	7.0	7.0	6	5.1	4.0
Histidine[e]	2	1.5	0.7	0	0	0
3-MeHis[e]			1.0			
Arginine	1	0.9	1.3	2	1.4	1.0

[a] Molar ratios of amino acids were calculated by assuming aspartic acid to be 13 residues. [b] Molar ratios of amino acids were calculated by assuming aspartic acid to be 9 residues. [c] Numbers were counted from the figures in Matthews *et al.* (1967). [d] Uncorrected for recovery with hydrolysis time, 20 hr. [e] Data were obtained by using a PA-35 column and were uncorrected.

placed in an 8 M urea solution. If sulfonylation had occurred, the spectrum would be changed by a *p*-nitrobenzene chromophoric group as shown by Gold and Fahrney (1964) and by Kallos and Avatis (1966). It has been confirmed experimentally in this work that the addition of *p*-nitrobenzenesulfonic acid to native chymotrypsin at an equimolar ratio causes the λ_{max} to shift from 281 to 270 mμ. *p*-Nitrobenzenesulfonic acid was not observed when two-dimensional chromatograms of acid hydrolysates of the modified enzyme were examined under ultraviolet light.

Further support for the methylation of an active site comes from the amino acid analyses and radioactive methylation results. Amino acid analyses of the modified chymotrypsin and its B chain showed that one histidine residue is converted into 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid. The radioactive methyl group combined stoichiometrically with the enzyme and the radioactivity was found in the B chain.

The spot which corresponds to histidine-57 in the peptic digestion showed a faint color reaction to the Pauly reagent after high-voltage electrophoresis. (The change of pK by methylation at N-3 of imidazole ring is probably small, then after methylation of histidine-57 its position on the electropherogram did not change significantly.) This indicates that mainly histidine-57 has been methylated. The radioautogram of peptic digestion of the modified α-chymotrypsin showed strong radioactivity in the area of the modified histidine-57, and much less radioactivity in the region of histidine-40. A very small amount of radioactivity was also found in the area of tyrosine-171.

Recently Blow *et al.* (1969) proposed that N-1 of histidine-57 is in a buried position stabilized through hydrogen bonding by aspartic-102. N-3 of histidine-57 would participate in the catalysis with serine-195 as a "charge relay system." Thus methylating histidine-57 at N-3 position should cause the enzyme to lose its catalytic activity. The fact that N-3 of histidine-57 is selectively alkylated by our reagent on the enzyme is in satisfying agreement with the X-ray data of Blow *et al.*

A minor modification of histidine-40 and tyrosine-171 was also observed. These groups were methylated slowly and randomly by the methyl group of the modifying reagent.

The hydrolysis rate of the methyl ester was not affected by addition of functional amino acids of chymotrypsin except by high concentration (5.00×10^{-2} M) of imidazole ($k_2/K_s = 2.2 \times 10^{-3}$ M^{-1} sec^{-1}). Even this rate is low compared with the inhibition rate of chymotrypsin by the methyl ester ($k_2/K_s = 41.5 \times 10^{-2}$ M^{-1} sec^{-1}): the inactivation of chymotrypsin by the methyl ester is at least 200 times faster than the hydrolysis rate of the methyl ester under nonenzymic conditions. Therefore we assume that adsorption of the methyl ester on chymotrypsin has to occur before the modification takes place and that the methyl ester must be oriented favorably at the active site for the enzymatic catalysis. When histidine in pH 7.9 buffer solution was mixed with the methyl ester at 25° for 48 hr, 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid was formed preferentially over 2-amino-3-(1-methyl-4-imidazolyl)-propanoic acid. In contrast, only 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid was obtained by the methylation of α-chymotrypsin. Since the methylation reaction is undoubtedly an SN2 process—usually not susceptible to catalysis—these results indicate that the nitrogen at the 3 position

of the histidine moiety is the function responsible for general base catalysis in the action of chymotrypsin. This is further supported by the finding that the catalytic activity of the enzyme is decreased by at least three powers of ten upon methylation of N-3 of histidine. Thus, this experiment defines the detailed stereochemistry of histidine-57 with respect to the active site of chymotrypsin and the nonequivalence of the two nitrogens of the crucial imidazole ring. A similar nonidentity of the two nitrogens of the catalytical histidines of ribonuclease has been reported (Heinrikson *et al.*, 1965; Heinrikson 1966). The role of the nitrogen at position 1 remains to be elaborated.

Methyl *p*-nitrobenzenesulfonate is a prototype of a new class of active-site-specific reagents which are characterized by the fact that the group directing the reactive function to the active site is eliminated as the desired modification takes place.

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