Alkylation of Amide Linkages and Cleavage of the C Chain in the Enzyme-Activated-Substrate Inhibition of α-Chymotrypsin with *N*-Nitrosamides†

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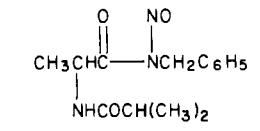
ABSTRACT: Active-site-directed *N*-nitrosamides inhibit α-chymotrypsin through an enzyme-activated-substrate mechanism. In this work, the activation results in the release—in the active site—of benzyl carbonium ions, which alkylate and inhibit the enzyme. The final ratio of benzyl groups to enzyme molecules is 1.0, but the alkyl groups are scattered over a number of sites. Reduction and alkylation of the inhibited enzyme generate peptides insoluble in most media. Guanidine hydrochloride at 6 M proved a good solvent, and its use as an eluant on G-75 Sephadex permitted separation of the peptides. In the case of ^{14}C-labeled enzyme, such an approach has shown that all of the alkylation occurs on the C chain of the enzyme, the chain of which the active site is constructed. Chemical modification of the peptides with ethylenediamine and *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide rendered them soluble in dilute acid, permitting high-performance liquid chromatographic separation. Model studies have shown that the benzyl carbonium ions are highly reactive, alkylating amide linkages at both oxygen and nitrogen. Alkylation at oxygen produces imidate esters, which are labile centers. Hydrolysis of protein imidates results in a cleavage of the chain at that point, and separation of the peptides formed (followed by analysis) permits their identification. In our inhibition of α-chymotrypsin, a major site of O-alkylation has been identified as the carbonyl oxygen of Ser-214. Alkylation at the nitrogen atom of amide linkages generates stable labels; full hydrolysis with 6 N HCl then leads to *N*-benzyl amino acids characteristic of those sites. Chromatography of this mixture and also ^{13}C NMR spectroscopy of the intact inhibited enzyme have shown that three major N-alkylations have occurred. Tryptic digestion of the C chain of chymotrypsin, which contains all of the alkylation sites, provides evidence that the stable N sites are principally located between residue 216 and residue 230. These locations are consistent with predictions of alkylation sites based on inspection of a molecular model of chymotrypsin, with special reference to the aromatic binding pocket.

The enzyme α-chymotrypsin is rapidly and irreversibly inhibited by the active-site-directed enzyme-activated substrates D-*N*-nitroso-*N*-benzyl-*N'*-isobutyrylalaninamide (**1**), D-*N*-



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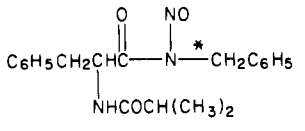

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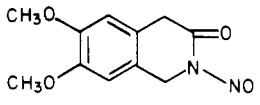

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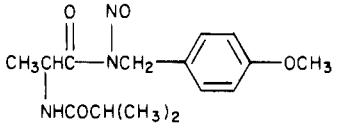

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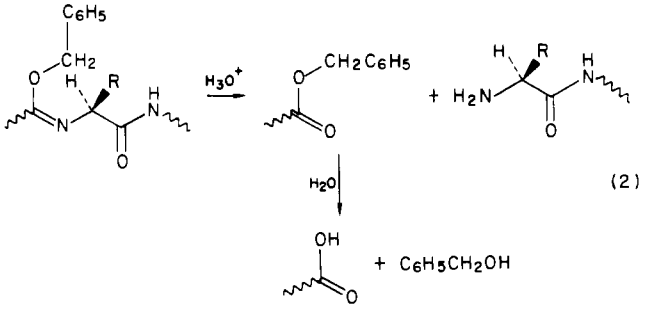
nitroso-*N*-benzyl-*N'*-isobutyrylphenylalaninamide [**2**; asterisk (*) = ^{12}C, ^{13}C, or ^{14}C], and *N*-nitroso-1,4-dihydro-6,7-dimethoxy-3(2*H*)-isoquinolinone (**3**) (White et al., 1975, 1977, 1978, 1981). Inhibition results when the enzyme is alkylated by the benzyl carbonium ions produced (eq 1); fully inhibited

$$2 \xrightarrow{\alpha\text{-chymotrypsin}} \text{isobutyryl-Phe–O-Ser-Enz} + C_6H_5CH_2N{=}N\text{-}O^- \rightarrow \rightarrow C_6H_5CH_2^+ \xrightarrow{\alpha\text{-chymotrypsin}} \text{benzylchymotrypsin} \quad (1)$$

α-chymotrypsin has a benzyl/enzyme ratio of approximately 1 (White et al., 1981).

The analogous alkylation of peptides, in model studies, has shown that deaminatively produced benzyl carbonium ions are sufficiently reactive to alkylate amide linkages and that both O- and N-alkylations occur (ratio ~5/1) (White et al., 1977; J. P. Cousins, unpublished work). In the enzyme inhibition, side-chain functional groups could, in principle, be alkylated (i.e., HO, H_2N, and RS groups and aromatic or heterocyclic rings), but the walls of the active site are constructed largely of amide groups, and reaction of the amide linkage should be favored with highly reactive reagents. Thus, in the carbonium ion inhibition of chymotrypsin, we anticipated alkylation of amides at oxygen, alkylation of amides at nitrogen, and alkylation of side chain groups—in the order of decreasing probability. The amide sites involving oxygen are of particular interest in that they represent centers of lability (Pletcher et al., 1968; Chaturvedi & Schmir, 1968); mild hydrolysis should cleave the peptide chains at those points (eq 2) and partial



(2)

sequencing of the new termini should identify the specific amino acid sites of alkylation. The carbonium ion approach

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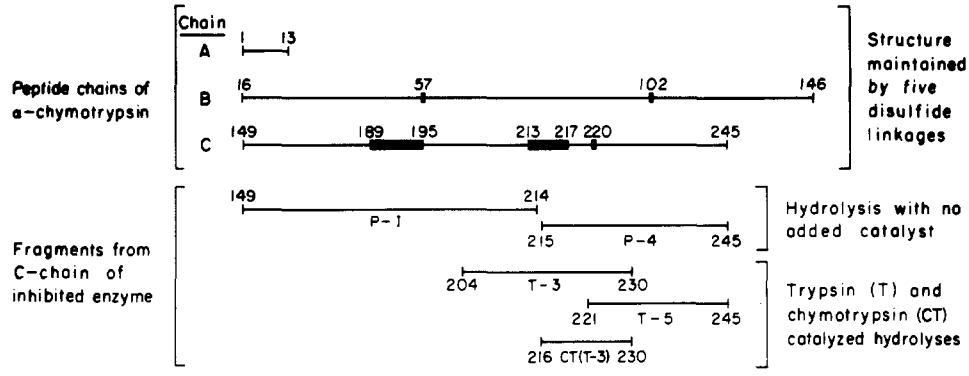


FIGURE 1: Schematic representation of the peptide chains of α-chymotrypsin and derived cleavage products. The numbers marking the chain are residue numbers (based on chymotrypsinogen). The thickened lines of the B and C chains represent the principal amino acids making up the aromatic binding region of the active site (Blow, 1976).

to inhibition thus has promise as a method that will map the active site of α-chymotrypsin with respect to the site of release of the carbonium ion. No other method of inhibition of enzymes (Sinnott, 1982; Silverman & Hoffman, 1984) involves intermediates powerful enough to react directly with amide linkages, with the exception of photoaffinity labelling (White et al., 1978; Bayley & Knowles, 1977). The amide linkages of proteins have been previously utilized in chemical reactions but only through intramolecular interactions (Glazer, 1976).

A second advantage of the high reactivity of deaminatively formed carbonium ions is that alkylation occurs at or very near the site of release. In other methods of affinity and "suicide" inhibition, the alkylating agents are relatively stable and selective; species of this type can bypass less reactive groups such as the hydroxyl group of Ser and Thr, only to react with better nucleophiles at some distance from the release site (Walsh, 1979).

α-Chymotrypsin is constructed of three peptides (A, B, and C chains) linked by disulfide bonds (Figure 1). With the exception of B-chain residues His-57 and Asp-102, the active site of chymotrypsin is constructed of C-chain residues (Blow, 1971, 1976). To allow isolation of the C chain and peptides generated from imidate hydrolyses (eq 2), the disulfide bonds of the inhibited enzyme were reduced, and the thiol groups produced were alkylated to block the re-formation of disulfide bonds by air oxidation. This paper describes (1) methods for overcoming the insolubility of peptides derived from α-chymotrypsin, (2) methods for separating peptides derived from chymotrypsin under conditions that avoid aggregation, (3) evidence that the nitrosamide inhibition of chymotrypsin leads to alkylation of the carbonyl oxygen of Ser-214 with the subsequent cleavage of the C chain at that point, and (4) an approach to the identification of the stable sites of alkylation (at N, S, and C) of ^{14}C-labeled and inhibited α-chymotrypsin.

Experimental Procedures

Materials

α-Chymotrypsin (type I-S), Gdn-HCl[1] (grade I), DTT, and IAA were purchased from Sigma, and D-tryptophan methyl ester, DFP, EDC, BTEE, TPCK, and ethylenediamine hydrochloride were Aldrich products. CH-Sepharose 4B and Sephadex (all types and grades) were obtained from Pharmacia. Trypsin was obtained from Nutritional Biochemicals. Dithiothreitol and all materials required for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were purchased from Bio-Rad. BDH Biochemicals provided cyanogen bromide fragments of myoglobin. DFP-inhibited chymotrypsin was obtained from ICN Pharmaceuticals. The sequencing reagents and solvents and phenylthiohydantoin amino acid standards were from Beckman Instruments. Carboxypeptidase Y, constant-boiling HCl, and pH 2.2 citrate buffer were products of Pierce Chemical Co.

Methods

Syntheses. The syntheses of inhibitors **1–3** (^{12}C and ^{14}C) have been described (White et al., 1981). The syntheses of ^{13}C-labeled **1** and **2** ($N^{13}CH_2C_6H_5$) and the 4-methoxy derivative of **2** ($NCH_2C_6H_4OCH_3$) followed the same procedures.

Removal of Contaminating Peptides from Chymotrypsin. α-Chymotrypsin was affinity purified on CH-Sepharose 4B–D-tryptophan methyl ester according to Pharmacia literature (*Affinity Chromagraphy, Principles and Methods*, 1977); the retained enzyme was eluted with 0.1 M AcOH, 0.002 M in $CaCl_2$, at pH 3.0. To remove small peptides before reduction and alkylation, the inhibited enzyme after dialysis and lyophylization was dissolved in 6 M Gdn-HCl at pH 3 and subjected to gel filtration on Sephadex G-25 sf, eluting with 0.02 M AcOH (modification of the Yapel et al. (1966) procedure; Hamilton & Zerner, 1981). A few fractions (<5%) absorbing in the UV range at 215 nm were detected for both DFP- and nitrosamide-inhibited enzymes between the protein and salt peaks.

Inhibition of α-Chymotrypsin by Nitrosamides **1–3** *and by DFP.* Affinity-purified enzyme was diluted to 3×10^{-5} M with a 0.1 M AcOH–0.002 M $CaCl_2$ buffer at pH 3, with a molar extinction coefficient of 52×10^3 cm^{-1} M^{-1} at 282 nm being used to determine concentration (Wilcox, 1970). The pH of the solution was then brought to 7.9 with 3 M Tris. The inhibitor in CH_3CN was added dropwise over a 7–8-min period with stirring [final ratio of inhibitor:enzyme = 10:1 (mol/mol); final CH_3CN content = 10% in volume]. The mixture was assayed for chymotryptic activity with BTEE (Hummel, 1959). After 20–25 min, DFP was added (10:1 molar ratio to the enzyme). After an additional 20 min, 6 N HCl was added to pH 3, and the turbid solution was filtered through Whatman No. 1 paper. The filtrate was dialyzed for 24 h at 4 °C vs. 1 mM HCl (3 times 500-mL baths), and the dialyzed enzyme was lyophilized. For ^{14}C inhibition, 10 equiv of [^{14}C]-**2** (sp act 2.4×10^3 dpm/nmol) was used. For DFP inhibition alone, 10 equiv of DFP was added to the enzyme after the pH had

[1] Abbreviations: DTT, dithiothreitol; IAA, iodoacetic acid; DFP, diisopropyl fluorophosphate; BTEE, benzoyltyrosine ethyl ester; TFA, trifluoroacetic acid; Gdn-HCl, guanidine hydrochloride; EDC, *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; PTH, phenylthiohydantoin.

been brought to 7.9, and the subsequent workup was the same as that described above; the DFP reaction mixture did not develop turbidity.

Reduction and Alkylation of Chymotrypsin. Under a blanket of N_2, the enzyme was dissolved at a concentration of 5–10 mg/mL in a freshly degassed buffer of 7 M Gdn-HCl, 0.1 M Tris, and 0.002 M ethylenediaminetetraacetic acid adjusted to pH 9.1 with glacial acetic acid (Stanton & Viswanatha, 1971; except that Gdn-HCl was used). To this solution was added a 20-fold excess of DTT (relative to total disulfide bonds). The reaction was maintained for 4 h at room temperature; then, iodoacetic acid (1.5 mol/SH equivalent) and the same weight of solid Tris were added under nitrogen (Hexter & Westheimer, 1971). After 20 min, the reaction was quenched by addition of an amount of β-mercaptoethanol equivalent to the iodoacetic acid added (the mixture was kept in darkness until utilized).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gels were prepared and run according to the procedure of Swank & Munkres (1971), except that deionized urea was used to prepare the gels to avoid carbamylation of peptides (Cole, 1961) (~1 g of Amberlite MB-3 was stirred for 30–60 min in about 40 mL of warm 10.7 M urea; the urea solution was filtered through a 0.45-μm Millipore filter into a warm suction flask). The stock solutions of Swank and Munkres were prepared at twice their specified concentrations so that upon addition to the urea solution the final concentrations were correct, and the urea concentration was 8 M. Reduced and alkylated protein was prepared for electrophoresis by desalting either by gel filtration on Sephadex G-15 or by repeated ultraconcentration in an Amicon concentrator over a membrane with a 1000-dalton cutoff. The desalted protein was lyophilized and dissolved in sample buffer. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 by the method of Swank & Munkres (1971). At least two gels in each run contained as standards the five cyanogen bromide fragments of myoglobin with molecular weights of 16 949, 14 404, 8159, 6214, and 2512.

Sephadex G-75 Chromatography in 6 M Gdn-HCl. The reduced and alkylated product mixture from chymotrypsin either was loaded directly onto a Sephadex G-75 sf column equilibrated with 6 M Gdn-HCl, 0.01 M in Na_2HPO_4, at pH 5 and eluted with the same solvent system or was desalted first on Sephadex G-15 with 0.05 M NH_4OH, lyophylized, dissolved in the Gdn-HCl system, and then chromatographed on Sephadex G-75, (2.6 × 89 cm and 1.2 × 106 cm columns, flow rates 2–4 mL/h).

Desalting Peptides from Chymotrypsin. Peptides obtained from Sephadex G-75 separation of reduced and alkylated chymotrypsin were desalted on Sephadex G-25 m or G-15 (2.5 × 32 cm columns) with 0.05 M ammonium hydroxide or ammonium acetate (pH 9) as eluants. Elution with acetic acid alone (0.05–0.1 M) or ammonium acetate (0.05–0.1 M) led to poor recoveries of peptides; these solvent systems were satisfactory for desalting intact chymotrypsin or chemically modified peptides, however. The conductivity of fractions was measured (a London Co. meter) in representative runs to choose the salt-free fractions for pooling.

Chemical Modification of Carboxyl Groups. A modification of the procedure of Carraway & Koshland (1972) was used; proteins or peptides (1–10 mg/mL in 5 M Gdn-HCl at pH 3, 1 M in ethylenediamine) were allowed to react with 0.1 M EDC at pH 4.75; manual addition of a few microliters of 1 N NaOH was sufficient to keep the pH constant. After 1 h, 5 volumes of 1 M AcONa at pH 4.75 was added, and the mixture was desalted on a Sephadex G-25 column (1.5 × 76 cm), eluting with 0.05 M AcOH. The protein derivative was then reduced and alkylated and desalted on a Sephadex G-25 column (1.7 × 50 cm), eluting with 0.05 M AcOH.

High-Performance Liquid Chromatography. The Waters Associates, Inc., system used consisted of an ALC 202 liquid chromatograph equipped with one 6000 A and one 6000 pump connected to a 660 solvent programmer and to a U6K sample injector. μBondapak phenyl columns (3.9 × 300 mm, Waters) were used principally (poor recoveries were experienced with μBondapak C_{18} columns). The mobil phase for peptide fragments from both native and chemically modified enzyme consisted of H_2O/CH_3CN (Waters HPLC grade) containing 0.1% (v/v) TFA (Fluka sequenal grade). Linear gradients were usually run at 1.0 mL/min. The eluates were monitored continuously with either a Beckman 25 spectrophotometer equipped with a Waters LC 25 microcell assembly or an ISCO 1840 adsorbance detector equipped with an ISCO 19-μL analytical flow cell.

Amino Acid Analyses. Peptides were hydrolyzed with 6 N HCl (Pierce, constant boiling) for 20 h at 115 °C and chromatographed on a Durrum-500 amino acid analyzer in the following Durrum citrate buffers in sequence: 0.2 N, pH 3.25; 0.2 N, pH 4.25; 1.0 N, pH 7.40. When tryptophan was to be determined, hydrolyses were performed in 4 N methanesulfonic acid for 20 h at 115 °C (Simpson et al., 1976). Chromatography was performed as above, or else, the third buffer was changed to 0.2 N and pH 5.88 to retard tryptophan.

Autoradiography. Amino acids were chromatographed on 8-in. square silica gel plates with a $CHCl_3/CH_3OH/17\%$ NH_4OH (2:2:1 v/v) eluant in the first direction and phenol/water (3:1 w/w) in the second. After being dried, the plate was covered with Saran wrap and exposed to Kodak XR-5 X-ray film at 4 °C for 70 h.

Radioactive Counting. ^{14}C-Labeled samples were dissolved in 5 mL of Hydrofluor (National Diagnostics) and counted on a Prias PLD liquid scintillation counter with an automatic program for quenching correction.

Automated N-Terminal Sequencing of Peptides. Approximately 5–10 nmol of peptide was subjected to automated Edman degradation with a Beckman 890C liquid-phase sequencer, using program no. 121078 with 0.1 M quadrol. Polybrene (5 mg) was added to the spinning cup and carried through five sequencing cycles before the peptide was added. Double coupling was used (heptafluorobutyric acid addition was omitted in the first coupling cycle). The anilinothiazolinones were converted into phenylthiohydantoin derivatives by a modification of the method of Tarr (1977) (heating under nitrogen at 65 °C for 10 min in 1.5 M HCl in methanol). The conversion medium was removed by vacuum evaporation in a centrifuge evaporator, and the phenylthiohydantoins were analyzed by high-performance liquid chromatography according to the procedure of Zimmermann et al. (1977). Phenylthiohydantoins were hydrolyzed to the original amino acids in nearly quantitative yields by the procedure of Lai (1977) for the hydrolysis of anilinothiazolinones.

Sequence Analysis with Carboxypeptidase Y. The carboxyl terminus of peptide P-1 (ca. 2–4 nmol) was determined at 0 °C at a peptide/carboxypeptidase ratio of ~100 (Hayashi, 1977; Hayashi et al., 1963; Kuhn et al., 1974; Martin et al., 1977). Peptide P-1 was digested by carboxypeptidase with unusual speed, and the digestion was conducted at 0 °C rather than the customary 25 or 37 °C so that the yields of released amino acids after short exposures could be differentiated. Angiotensin II and the C chain of chymotrypsin were analyzed

for their carboxyl termini as controls, angiotensin II at 20 °C at a peptide/carboxypeptidase ratio of 91:1 and chymotrypsin C chain at 37 °C at a peptide/enzyme ratio of 56:1. For all analyses, the concentration of carboxypeptidase was between 0.3 and 0.9 μM. At intervals, an aliquot (ca. 100 μL) of the reaction mixture was removed and boiled to inactivate the enzyme, and the following procedure was employed to remove ammonia, large amounts of which interfere with the analysis of Trp: lyophilization, dilution with 40 μL of water, lyophilization, adjustment of the pH to 11 with 0.06 M NaOH, lyophilization, dilution with 40 μL of water, lyophilization, and amino acid analysis. When 0.2 N citrate (pH 5.88) was used as the third buffer in the amino acid analyses, removal of ammonia was not necessary since under these conditions Trp emerges considerably ahead of ammonia.

Isolation of A Chain. TPCK-inhibited chymotrypsin (Schoellman & Shaw, 1963) was reduced and alkylated, desalted, and lyophilized. The residue was suspended in 0.05 M NH_4OH (about 90% dissolves), and after centrifugation, the supernatant was applied to a Sephadex G-75 column and eluted with 0.05 M NH_4OH, monitoring at 215 nm. The fractions containing a peak eluting at $2.0V_0$ were pooled, lyophilized, and analyzed. The amino acid analysis was normalized to Val = 2 [found (theory)]: Asx 0.1 (0), Ser 0.7 (1), Glx 1.1 (1), Pro 2.0 (2), Gly 1.72 (2), Ala 1.1 (1), Val 2 (2), Ile 0.8 (1), and Leu 1.6 (2). The material retrieved by gel chromatography could be further purified by HPLC (Rivier, 1978). The peptide was eluted after about 9 min in 26% acetonitrile–74% 0.25 M H_3PO_4 adjusted to pH 2.25 with triethylamine (flow rate 1 mL/min). The A chain could also be secured by TLC on silica gel G plates with $CHCl_3$–methanol–ethanol–concentrated NH_4OH (2:1:1:1 v/v) as the eluant, according to the method of Fishbein et al. (1980).

Carbon-13 NMR Spectra. Samples were run in 3.0 mL of 90% H_2O/10% D_2O at pH 0.76 with acetonitrile as an internal reference (120.49 ppm from external Me_4Si). The carbon-13 NMR spectra were acquired on a Varian Associates XL-400 spectrometer at 100.563-MHz resonance frequency for ^{13}C. Quadrature detection was employed with a pulse width of 17.5 μs (83°), and acquisition time was 1.0 s with no delay. Waltz-16 decoupling was used to decouple the protons from the carbon resonances. The sample temperature was 37 ± 1 °C, and about 65 000 transients were acquired. A line broadening of 5 Hz was employed in the Fourier transformation.

Results and Discussion

Inhibition of α-Chymotrypsin by Nitrosamides **1** *and* **2**. Chymotrypsin was 90%–95% inhibited within 10 min on treatment with 10 equiv of nitrosamide **1** (or **2**). DFP was then added to prevent autolysis by any residual active enzyme; within 10 min, no detectable activity (0%–0.1% of the original value) was observed with BTEE. α-Chymotrypsin inhibited with DFP alone was used as a control for autolysis and for tightly bound peptides possibly present in the native enzyme.

The full inhibition of α-chymotrypsin by D-**1** was paralleled by the covalent attachment of benzyl groups to the protein. After dialysis, the [^{14}C]-**2**-inhibited enzyme contained 1.05 benzyl groups/molecule.

Reduction and Alkylation of α-Chymotrypsin. Alkylation of the reduced enzyme with iodoacetic acid was complex. Alkylation for 20 min resulted in the appearance of two bands on SDS–polyacrylamide gels representing the B and C chains (see below). Enzyme alkylated for longer times showed three to seven bands in the same molecular weight region, yet amino acid analyses still showed the theoretical 10 (carboxymethyl)cysteines. Overalkylation may lead to the introduction of additional charges on the protein by sulfonium ion formation with Met and CM-Cys, but since amino acid analyses gave the correct value for CM-Cys (10) and Met (2), the overalkylation must be reversed during the hydrolysis step prior to amino acid analysis.

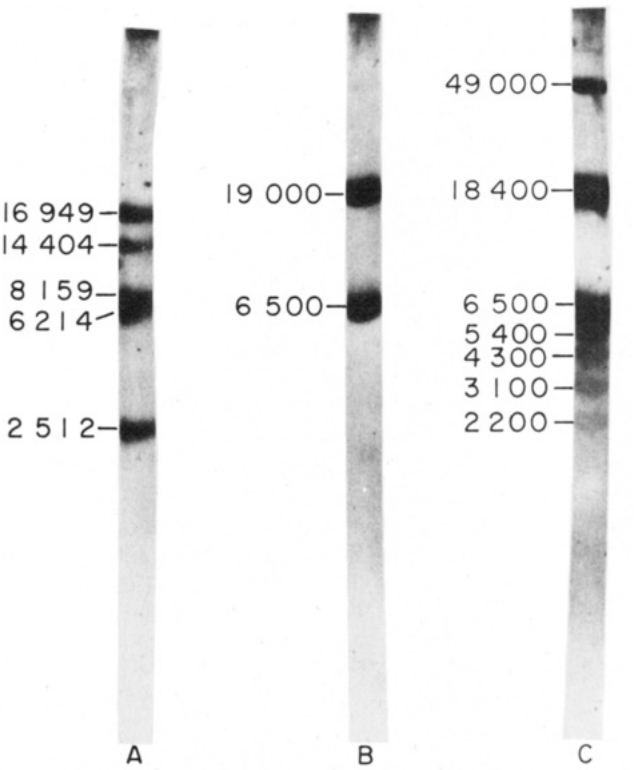


FIGURE 2: SDS–polyacrylamide gel electrophoresis of (A) myoglobin fragments, (B) reduced and alkylated α-chymotrypsin, and (C) reduced and alkylated nitrosamide **2** inhibited α-chymotrypsin.

Electrophoresis. Gel electrophoresis (SDS–PAGE) of the reduced and alkylated native and DFP-inhibited chymotrypsins showed the B and C chains (Figure 1) as major bands with apparent molecular weights of 19 000 and 6400, respectively (±5%) (Figure 2B) (similar bands were seen when the intact enzyme was electrophoresed in a buffer containing 2-mercaptoethanol); identification stemmed from the amino acid analyses (Table I, e.g.) and from electrophoresis of the individual chains separated by Sephadex chromatography (see below). A faint high molecular weight band was also noted with an apparent molecular weight of ca. 40 000–50 000. The material responsible for this band, isolated by Sephadex G-75 chromatography, gave an amino acid composition corresponding to the sum of the B and C chains. The A chain was not normally observed by SDS–PAGE, presumably because it diffuses from the gel during staining and destaining and/or it does not strain well enough to be visualized (Dunker & Rueckert, 1969). The molecular weights derived from the gels do not correspond well with the actual molecular weights of the proteins (B chain, 13 390; C chain, 10 700; intact enzyme, 25 260) (Brown & Hartley, 1966). Anomalous molecular weights for small peptides can be attributed to small variations in conformation and intrinsic charges (Swank & Munkres, 1971; Dunker & Rueckert, 1969) and for larger peptides to altered binding of SDS as a consequence of incomplete denaturation (due to the presence of disulfide bonds or to other structural features) (Abernathy et al., 1974; Reynolds & Tanford, 1970; Fish et al., 1970).

In contrast to gels of the native and DFP-inhibited chymotrypsins, which showed, essentially, only bands for the B

Table I: Amino Acid Composition of B and C Chains Obtained from DFP-Inhibited Chymotrypsin and of ^{14}C-Labeled C Chain Obtained from ^{14}C-Labeled D-Phe-2-Inhibited Chymotrypsin

	B chain			C chain			
residue	Sephadex G-75[a,b]	HPLC[c,d]	theory[e]	Sephadex G-75[a,b]	HPLC[c,d]	^{14}C labeled[a,d]	theory[e]
CM-Cys	2.9	3.8	4	4.5	4.6	5.4	5
Asx	12.7	13.2	13	8.6	8.7	8.9	9
Thr	14.3[g]	12.6	13	10.4[g]	8.1	8.5	9
Ser	15.0[g]	13.6	15	10.2[g]	9.7	10.0	11
Glx	10.3	10.2	10	4.4	4.6	4.2	4
Pro	3.1	2.9	3	3.8	3.9	ND[k]	4
Gly	11	11.4	11	9.5	9.6	10.4	10
Ala	11.1	11.6	11	10	9.6	10.4	10
Val	12.9	13.6	13	7.8	8.8	8.2	8
Met	0	trace	0	1.7	2.0	1.9	2
Ile	5.4	5.6	6	2.5	3.3	2.8	3
Leu	9.2	9.0	9	7.7	8.1	8.1	8
Tyr	1.8	2.8	2	1.7	1.9	1.9	2
Phe	5.8	5.7	6	0	0	0	0
53.0[h]						0.1	
54.2[h]						0.1	
His	2.0	2.5	2	0	0	0.1[i]	0
Lys	7.5	8.7	8	5.0	6.4	6.1	6
Arg	0.9	0.8	1	1.8	2.1	2.0	2
Trp[j]	ND	3.9	4	ND	4.1	4.0	4

[a] Obtained as described in Figure 3. [b] Values normalized to Gly = 11. [c] Obtained as described in Figure 4. [d] Values normalized to best fit for Asx, Glx, Gly, Ala, Leu, and Lys. [e] Brown & Hartley (1966). [f] Values normalized to Ala = 10. [g] Corrected (Moore & Stein, 1963). [h] Extra peaks observed in ^{14}C-labeled C chain, reported as retention times (min); amount was calculated by assuming His standard color. [i] Suspected extra peak in ^{14}C-labeled C chain (see text). Refer to footnote *h*. [j] Calculated from adsorbance at 280 nm before hydrolysis, subtracting the contribution of Tyr. Molar adsorptivities for Trp and Tyr are from Bredderman (1974). [k] Not determined.

and C chains, nitrosamide-inhibited α-chymotrypsin (Figure 2C) showed bands for four smaller peptides with apparent molecular weights of ca. 5500, 4300, 3100, and 2200 in addition to bands for the B and C chains. Further, the high molecular weight band (40 000) representing linked B and C chains was much more intense in gels of the benzyl enzyme than in gels of the native and DFP enzymes.

Separation of B and C Chains of Chymotrypsin. The B and C chains of chymotrypsin (Figure 1) are insoluble in most solvents, and they exhibit a strong tendency to aggregate in the few low-salt solvent systems that will dissolve them. Reported approaches to a solution of this problem involve the use of ion-exchange chromatography in 8 M urea (Hartley, 1963; Van Hoang et al., 1963; Nakagawa & Bender, 1970; Hexter & Westheimer, 1971) and in 4 M urea (Bridges & Knowles, 1974), fractional precipitation from urea and SDS solutions (Richmond, 1966), selective dissolution in 0.1 M borate followed by ion-exchange chromatography (Glauser & Wagner, 1965), and chemical modification with succinic anhydride (Hapner & Wilcox, 1970) and with ethyleneimine (Burkhardt & Wilcox, 1967).

Chromatography on DEAE-cellulose of the sulfite-cleaved chains of DFP- and nitrosamide-inhibited chymotrypsin according to the method of Van Hoang et al. (1963) was not useful for our purposes in that a peak for the B chain was well resolved, but the C-chain eluted with the solvent front and cleavage peptides were not resolved. Varying the pH and salt concentration of buffers was unsuccessful. We initially investigated gel filtration procedures that would require only lyophilization for the final isolation step. Ammonium hydroxide (0.05 M) is capable of dissolving about three-fourths of the reduced and alkylated chains of chymotrypsin. However, chromatography in this solvent on Sephadexes G-75 sf and G-100 sf led to aggregation of the B and C chains, as evidenced by a void volume peak that showed both the B and C chains (by SDS–PAGE). The two chains were also eluted individually after the void volume, but each was contaminated by the other chain. Chromatography in the denaturing agent 8 M urea (Belew et al., 1978) was carried out on Sephadex G-50 sf, but no resolution of the B and C chains was achieved. To avoid carbamylation of peptides by ionic contaminants in urea (Cole, 1961) Gdn-HCl was then used (D'Alessio et al., 1975; Eipper & Mains, 1979). Gdn-HCl at 6 M is an excellent solvent for the peptides derived from α-chymotrypsin, and chromatography on Sephadex G-75 in the presence of Gdn-HCl led to a useful level of resolution (Figure 3). The first peak, a relatively small one eluting at $1.1V_0$, had an amino acid composition identical with the sum of the B and C chains, while the peaks eluting at $1.4V_0$ and $1.6V_0$ corresponded respectively to the B and C chains (Table I).

Chemical Modification of α-Chymotrypsin. The B and C chains of chymotrypsin and derived peptide fragments are only partially soluble in basic media such as 0.1 M ammonium hydroxide; they are almost totally insoluble in the acidic media most conveniently used for HPLC separation of peptides [trialkylammonium phosphates at pH 2.22 (Rivier, 1978) or 0.1% (v/v) TFA in aqueous acetonitrile (communication from Waters Associates, Inc., Milford, MA)]. To achieve the desired solubility, chymotrypsin was chemically modified to increase the number of positive charges on the molecule in acidic media by condensing carboxyl groups of the enzyme with ethylenediamine. This approach had been used to increase the solubility of TMV protein [Budzynski & Means, 1971; for a related case, see Carraway et al. (1969)]. In the present case, lyophilized peptides obtained from such chemically modified chymotrypsin dissolved readily in 0.1% TFA.

The chemically modified DFP-inhibited chymotrypsin was reduced and alkylated and subjected to HPLC on a reverse-phase phenyl column; the B and C chains were cleanly separated (Figure 4) and recovered quantitatively. The first peak, with a retention time of 39 min, had the amino acid composition of the the B chain, while the second, with a retention time of 49 min, corresponded to the C chain (Table I). The slower elution of the C chain probably stems from the larger number of hydrophobic residues on the C chain compared to the B chain. Further, chemical modification with ethylenediamine can add 17 positive charges to the B chain but only 8 to the C chain.

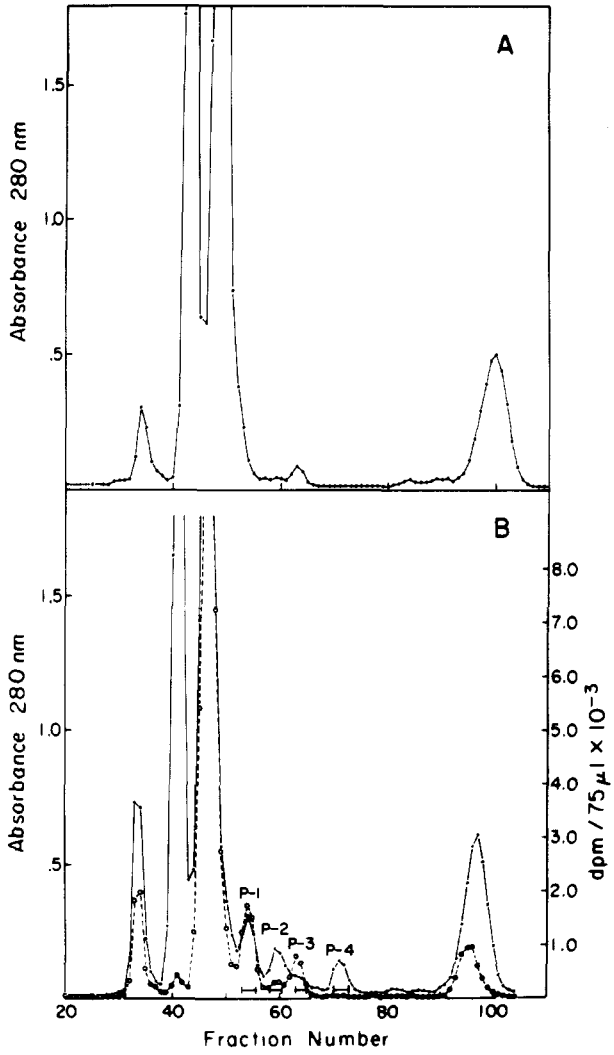


FIGURE 3: Gel filtration of peptides from α-chymotrypsin. DFP-inhibited (curve A) and [^{14}C]-**2**-inhibited (curve B) chymotrypsin were gel filtered after reduction and alkylation on a Sephadex G-75 sf column (1.2 × 106 cm) eluted with 6 M Gdn-HCl–0.01 M Na_2HPO_4 (pH 5) at 1.5 mL/h; 2-mL fractions were collected. Solid circles indicate adsorbance profile; empty circles with dotted lines indicate radioactivity (100 μL/fraction counted). Brackets indicate pooled fractions.

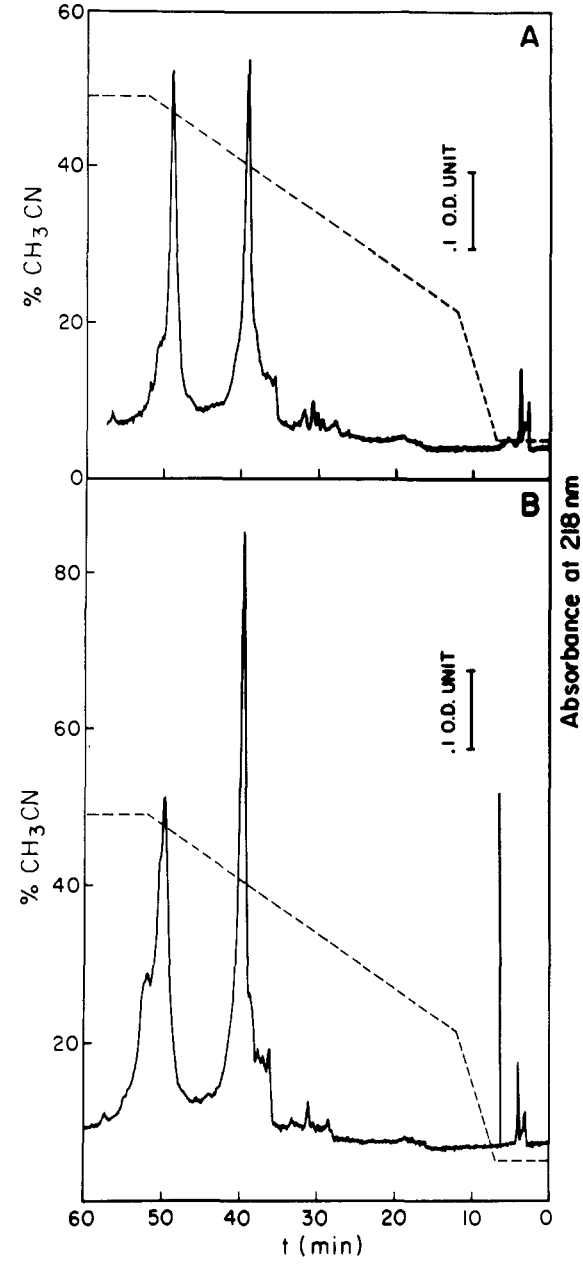


FIGURE 4: Reverse-phase HPLC of chymotrypsin chains and derived peptides. DFP-inhibited (curve A) and **2**-inhibited (curve B) chymotrypsin were chemically modified with ethylenediamine, reduced and alkylated, desalted, and injected onto a μBondapak phenyl column. The flow rate was 1.0 mL/min, and the mobile phase consisted of H_2O/CH_3CN containing 0.1% (v/v) TFA. The dotted line indicates the CH_3CN gradient.

For separation on Sephadex G-75 with volatile solvents, the modified peptides were loaded in 6 M Gdn-HCl and eluted with 0.1 M NH_4OAc at pH. 5. If the sample was not dissolved in Gdn-HCl-containing solvents (0.1 M NH_4OAc at pH 5 or 0.05 M AcOH), high molecular weight aggregates rich in the C chain were obtained. The presence of a cation (such as NH_4^+) in the eluant improved resolution, presumably by weakening the interactions between the polycationic peptides and the free carboxyl groups present on the gel. Surprisingly, the elution order of the B and C chains was reversed (the C chain, although of lower molecular weight than the B chain, eluted first).

Separation of Peptides from Inhibited Chymotrypsin. DFP-inhibited chymotrypsin and chymotrypsin inhibited with nitrosamide (**2**; ^{12}C and ^{14}C) were reduced and alkylated and gel filtered on Sephadex G-75 in 6 M Gdn-HCl (Figure 3). In the trace of the nitrosamide-inhibited enzyme (curve B), two additional peaks appear compared to the trace for DFP-inhibited enzyme (curve A): P-1, eluting at $1.8V_0$ (fraction no. 53), and P-4, eluting at $2.4V_0$ (fraction no. 71) (two smaller peaks, P-2 and P-3, also appear in the trace). A simultaneous decrease in the C-chain area (Table II) suggests that these fragments stem from the C chain. In our working hypothesis (eq 2), fragmentation of the C chain will occur when an *O*-benzylimidate group formed as a result of benzyl carbonium ion reaction with an amide linkage in the active site is hydrolyzed. It will be shown subsequently that P-1 is the N-terminal portion of the cleaved C chain (Figure 1) and that P-4 is the C terminal portion. P-1 carried a radioactive label, indicating that the C terminus was still in the form of a benzyl ester (eq 2). The label was rapidly lost on desalting at pH 10 (Table II); benzyl esters are known to be relatively stable at pH 3 but quite labile at high pHs (Tommila & Hinshelwood, 1938). P-4 is nonradioactive, as expected.

Another pair of fragments can be seen in Figure 3, one eluting at $2.0V_0$ (P-2) and the other at $2.2V_0$ (P-3). P-3 may be present in DFP-inhibited chymotrypsin also, albeit in lower quantity. In part, P-3 might be a peptide fragment tightly

Table II: Sephadex G-75 Chromatography in 6 M Gdn-HCl of Reduced and Alkylated DFP- and [^{14}C]-**2**-Inhibited Chymotrypsin[a]

	DFP enzyme	[^{14}C]-**2**-inhibited enzyme				
V_e/V_0[b]	area (%)[c]	area	dpm (%)[d]	retained dpm (%)[e]	benzyl group per molecule[f]	identity
1.1	2	7	7	66	0.60	B + C chains
1.4	48	43	2	60	0.01	B chain
1.6	47	39	71	90	0.75	C chain
1.8	t	5	7	38	0.12	P-1
2.0	<1	3	1	32	ND[h]	P-2
2.2	1	1	3	5	ND	P-3
2.4	0	3	0		0	P-4
3.2[g]			8	<1		salt volume

[a] Profiles given in Figure 3. [b] Elution volumes relative to void volume; latter determined with Blue Dextran. [c] Percent total area of total 280-nm adsorbance; areas calculated from manual integration of the eluate profile (Figure 3). [d] Percent of total radioactivity present in each peak. [e] Percent of recovered radioactivity retained in the peptide after desalting (Sephadex G-15 with 0.15 M NH_4OH elution), lyophilization, and a second desalting. [f] Benzyl group per molecule calculated from amino acid analysis of desalted peptides. [g] Adsorbance of this salt peak not considered for area calculations. [h] Not determined.

bound to the enzyme molecule and released upon reduction of the disulfide bridges; the higher radioactivity associated with it, however, suggests that this peak contained the C portion of one more pair of fragments coming from imidate hydrolysis (the N portion being the P-2 peak).

Peptide Separation after Chemical Modification. Resolution of the B and C chains of DFP-inhibited chymotrypsin on a μBondapak phenyl column (after chemical modification, reduction, and alkylation) was good (Figure 4A; amino acid analyses in Table I). The trace for nitrosamide **2** inhibited chymotrypsin was similar (Figure 4B), except for a decrease in height and a broadening of the C-chain peak, consistent with cleavage of that chain. No useful resolution of peptide fragments was observed, however. When P-1 and P-4 were isolated first on Sephadex G-75 in 6 M Gdn-HCl and then chemically modified, the percent acetonitrile required for their elution by HPLC corresponded to that for the region between the B and C chains. Thus, this approach while rapid and convenient was not useful as a separation method; it lended itself, however, to the rapid screening of inhibitors.

Nitrosamide **2** inhibited chymotrypsin (chemically modified, reduced, and alkylated) was also chromatographed on Sephadex G-75 with volatile eluants (0.1 M ammonium acetate). The peptide fragments were not resolved satisfactorily; P-1 and P-2 eluted as a single peak, and the resolution of P-3 and P-4 was insufficient.

Distribution of the ^{14}C Label. α-Chymotrypsin inhibited with [^{14}C]-**2** showed a benzyl/enzyme ratio of 1.0 after dialysis at pH 3 and also after gel filtration on Sephadex G-25 sf. When 6 M Gdn-HCl was used with the G-25 separation (Methods), about 5% of the ^{14}C was found associated with small peptides; these peptides, tightly associated with the active site (Hamilton & Zerner, 1981) but released on treatment with 6 M Gdn-HCl, are apparently capable of being alkylated by the benzyl carbonium ions generated from the nitrosamides.

After the enzyme was reduced and alkylated, the radioactive profile upon gel filtration on Sephadex G-75 in 6 M Gdn-HCl (Figure 3B) indicated that most of the radioactivity (more than 70%; Table II) is associated with the C chain, while the B chain has only a trace (less than 2%). This result is consistent with the view that alkylation occurs in the aromatic binding region of chymotrypsin, which is constructed of the C chain (Blow, 1971).

Some radioactivity (ca. 7%) was associated with the peak eluting at $1.1V_0$; this peak was more intense than that in the corresponding DPF trace (Figure 3) (7% vs. 2%, respectively; Table II). The $1.1V_0$ peak was found by amino acid analysis to correspond to the sum of the B and C chains; its specific activity, expressed as dpm per nanomole of C chain, indicated the presence of approximately one benzyl group per molecule. Cross-linking of the B and C chains probably occurs by means of amidine formation from an imidate group on the C chain (most probably the *O*-benzylimidate of Met-190)[2] and an amino group on the B chain [most probably the free amino group of Ile-14 (Hess, 1971)].[2] The resulting amidine enzyme could still be enzymically active. Among smaller fragments, radioactivity was associated mainly with P-1 and P-3, the other two fragments having almost no label.

Finally, some of the radioactive label (ca. 8%) eluted at the salt volume (13% if the prior Gdn-HCl G-25 separation is omitted). Upon successive desaltings, the substance responsible for the salt-volume radioactivity behaved as a small molecule, coeluting with guanidine hydrochloride on Sephadex G-15. The release from the enzyme of radioactivity as a small molecule (almost certainly benzyl alcohol, eq 2) indicates that some benzyl derivatives are labile under the conditions employed during the reduction and alkylation step (alkaline pH, high nucleophile concentration) but are resistant to mild acidic treatment (dialysis at pH 3); presumably, some buried imidates are exposed to the medium only during a complete unfolding of the enzyme molecule, such as during the reduction and alkylation.

In order to analyze the peptides obtained from Sephadex G-75 in 6 M Gdn-HCl, a desalting step was necessary. Table II shows the fate of radioactivity upon desalting with 0.05 M NH_4OH as the eluant. While the release of radioactivity was minimal for the C chain, most of the radioactivity associated with the peptide fragments was removed and coeluted with guanidine in the salt volume. These results were obtained with either 0.05 M NH_4OH or 0.05 M NH_4OAc (pH 9) as the eluant, and presumably, alkaline hydrolysis or ammonolysis of a benzyl ester occurred. Attempts to isolate the benzyl esters by using a desalting step at lower pHs (ca. 5) failed because of a poor recovery of peptides under those conditons. About two-thirds of the radioactivity in P-1 was lost during the desalting; the remainder was lost on hydrolysis with 6 N HCl and lyophylization.

Analyses of P-1 and P-4. The amino acid composition of P-1 (Table III) is consistent with the known sequence of residues 149–214 in chymotrypsin, and the amino acid composition of P-4 (Table III) is consistent with the known sequence of residues 216–245. A sample of peptide P-1 (8 nmol) was sequenced by the automated Edman degradation method for four steps. The second and fourth sequence steps of P-1 were identified as PTH-asparagine and PTH-proline, respectively. PTH-alanine was identified in the first sequence step (accompanied by an impurity that was not a PTH amino acid; after back-hydrolysis, the only amino acid recovered was alanine). No PTH amino acid was observed in the third sequence step, thus implicating a labile PTH amino acid. The sequence Ala-Asn-X-Pro, where X represents a labile PTH amino acid, can be assigned to the N-terminal sequence of P-1. The amino acid that yielded the labile PTH amino acid

[2] Established from a computer-based display of α-chymotrypsin using coordinates from the Protein Data Bank (Brookhaven National Laboratory). We thank Dr. L. M. Amzel, Interactive Graphics Facility, The Johns Hopkins Medical School, Baltimore, MD, for providing this service. See also Kraut (1971).

Table III: Amino Acid Composition of Peptide Fragments P-1 and P-4

residue	P-1[a,b] found[c]	P-1 theory (residues 149–214)[d]	P-4 found[e]	P-4 theory (residues 215–245)[d]
CM-Cys	3.3	4	0.9	1
Asx	6.1	7	2.0	2
Thr[f]	3.9	4	4.2	5
Ser[f]	5.7	7	3.1	4
Glx	3.1	2	2.3	2
Pro	3	3	1.2	1
Gly	8.1	8	2.6	2
Ala	6.0	6	4	4
Val	4.5	4	3.3	4
Met	1.5	2	0.1	0
Ile	2.6	3	0.5	0
Leu	5.4	6	2.3	2
Tyr	0.8	1	0.9	1
Phe	0.4	0	0.2	0
His	0.4	0	0.1	0
Lys	5.3	6	0.5	0
Trp[g]	1.9	2	0.8	2
Arg	0.6	1	0.8	1

[a] N-terminus: H_2N-Ala-Asn-Thr-Pro. [b] C-terminus: Gly-Ile-Val-Ser-CO_2H. [c] Normalized to Pro = 3. [d] Brown & Hartley (1966). [e] Normalized to Ala = 4. [f] Corrected (Moore & Stein, 1963). [g] Calculated according to Simpson et al. (1976).

was determined to be threonine by observation of α-aminobutyric acid as the product of back-hydrolysis of the product of the third sequence step (Lai, 1977). On the basis of this sequence, P-1 can only be the N-terminal portion of the C chain, which has the N-terminal sequence H_2NAla-Asn-Thr-Pro- (each four-residue sequence in α-chymotrypsin is unique).

The sequence of P-1 at the carboxyl terminus was determined by digestion with carboxypeptidase Y. This approach applied to angiotensin II and to the C chain of chymotrypsin led to the correct sequences. The release of amino acids from P-1 (2.4 nmol) in a 100:1 ratio to carboxypeptidase Y is shown in Figure 5. In the first minute the following amino acids were released in order of decreasing abundance: serine, valine, isoleucine, and glycine. After 30 min, leucine appeared, accompanied by further release of valine. The pattern of amino acid release is consistent with the sequence -Leu-Val-Gly-Ile-Val-Ser-COOH, which matches the sequence of chymotrypsin from leucine-209 to serine-214.

Two samples of P-4 (6 and 3 nmol) were sequenced by the automated Edman degradation method for four steps. However, no PTH amino acid was detected in the first four sequence steps, indicating a sequence of four labile amino acids or a peptide blocked at the amino terminus. The former possibility was eliminated from consideration when back-hydrolysis of the material isolated from the four degradation steps failed to release any known amino acid.

The results suggest that P-1 and P-4 are a pair of fragments arising from hydrolysis of an imidate formed with the carbonyl group of Ser-214; each should contain one tyrosine and two tryptophan residues, and Trp-215 should be the N-terminus of P-4. Hydrolysis with 4 N methanesulfonic acid was used for tryptophan determinations (Simpson et al., 1976); 1.9 residues/molecule were obtained from P-1, and 0.8 residue/molecule was obtained for P-4. Spectrophotometric determination of tryptophan (Bredderman, 1974) gave an ambiguous Trp/Tyr ratio of 1.6 for P-4, whereas calculations based on the known extinction coefficients of Trp and Tyr and an estimate of the concentrations based on the amino acid analysis of P-1 and P-4 gave a value of 2.

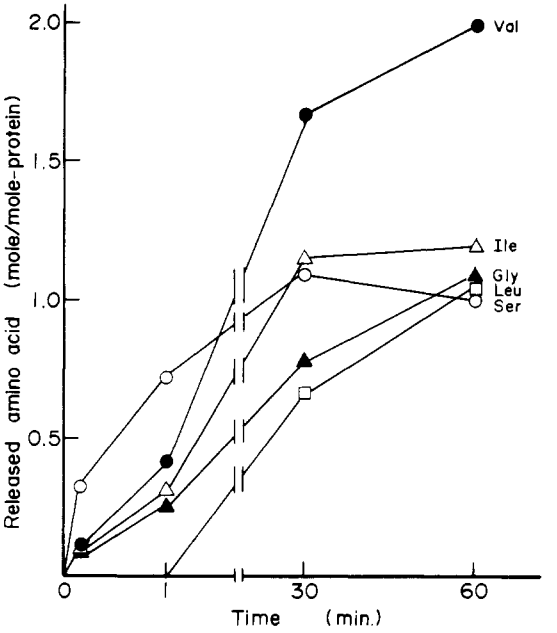
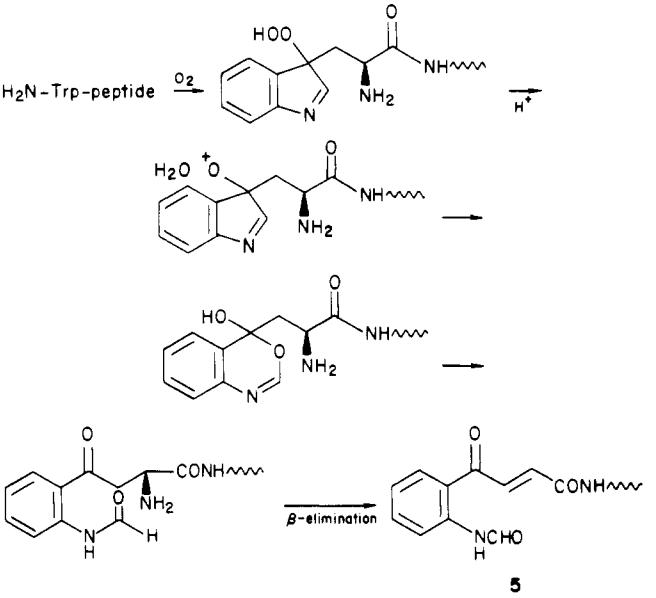


FIGURE 5: Release of amino acids from P-1 upon digestion with carboxypeptidase Y. The peptide (2–4 nmol) in 0.1 M pyridine–acetate buffer (pH 5.5) at a concentration of 10^{-4}–10^{-5} M was treated at 0 °C with 0.01 molar equiv of the enzyme (Methods).

The shortage of the tryptophan residue (amino acid analysis) and the lack of a detectable N-terminus in P-4 suggests that N-terminal Trp in P-4 is chemically altered (probably oxidized) during workup. N-Terminal Trp may require very special handling conditions.[3] A reasonable series of reactions illustrating how the oxidation of N-terminal tryptophan could block sequencing by the Edman approach is given in eq 3;



(3)

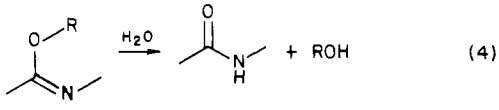
precedents exist for the formation of indole hydroperoxides (Beer et al., 1949a–c) and for the rearrangement of related hydroperoxides (Witkop & Patrick, 1951).

[3] We have been informed by Dr. Michael Hunkapiller (Division of Biology, California Institute of Technology) (March 10, 1983) that of all the peptides and proteins sequenced in his laboratory (Hunkapiller, 1983) none had Trp at the N-terminus. A similar conclusion for proteins has been communicated to us (June 17, 1984) by Dr. Russel F. Doolittle (Department of Chemistry, University of California, San Diego, CA). However, his data bank did list two small peptide degradation fragments (9 and 13 residues) that had Trp at the N-terminus.

Attempts To Increase Fragmentation. When the alanine-based nitrosamide **1** was used to inhibit chymotrypsin, HPLC analysis of chemically modified, reduced, and alkylated enzyme was similar to that obtained for the phenylalanine-based inhibitor **2** (Figure 4B), but the C-chain peak showed a less pronounced height decrease and its broadening was less relative to the peak from DFP-inhibited enzyme. Similarly, Sephadex G-75 separation with 6 M Gdn-HCl showed a pattern of peptide fragments similar to that of Figure 3B, but the yield of fragments was slightly lower. Thus, a change in the side chain attached to the α-carbon (CH_3– instead of $PhCH_2$–) only slightly affects the yield of fragments.

In the above studies, the low fragment yields isolated implied that only small quantities of imidates were present. From the Sephadex G-75 analysis (Figure 3B and Table II) and assuming that all the peptide fragments came from imidate hydrolysis, we can conclude from the extent of fragmentation detected that about 10% of the inhibited enzyme molecules contained a benzylimidate moiety. If we assume that all the radioactivity that was released during the reduction and alkylation steps and during successive desaltings came from hydrolysis of benzylimidates and esters and that all esters were formed upon acid hydrolysis of imidates, we can account for about 30% of the total radioactivity stemming from the O-benzylation of amides, while more than 70% appeared as stable *N*-, *S*-, and *C*-benzyl derivaties of the C chain. Thus, the minimum ratio of stable to labile benzyl groups arising from the alkylation of chymotrypsin was about 3:1. This result is considerably different from such ratios obtained in model studies; for example, the reaction of deaminatively formed benzyl carbonium ions with *N*-ethylacetamide led to an *N*-benzyl/*O*-benzyl ratio of 1/6 (White et al., 1978; J. Cousins, unpublished work). Either the active site is constraining the reacting molecules in some way, or the inhibition involves benzyl diazonium ions rather than benzyl carbonium ions in the relatively hydrophobic active site. The alkylations would then result from displacement reactions, and the high nucleophilicities of nitrogen (and sulfur) could well lead to low values of the alkylation ratio: oxygen/nitrogen (and sulfur).

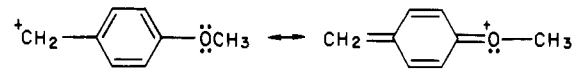
The desired hydrolysis of an imidate to the carboxylate ester and an amine salt (eq 2) occurs at low pHs; at high pHs, an alcohol and amide are the products formed (eq 4) (Pletcher



(4)

et al., 1968; Chaturvedi & Schmir, 1968). For our purposes (eq 2), such hydrolysis would not lead to chain cleavage, but instead to a regeneration of the protein (eq 4). To check whether this reaction pathway might have occurred at pH 7.9, the inhibition was carried out at a pH of 5. As expected (Himoe et al., 1967), the inhibition at pH 5 with **2** proceeded slowly; chymotrypsin was 70% inhibited by nitrosamide **2** in 80 min, compared to the 90% inhibition observed within 10 min at a pH of 7.9. The inhibited enzyme, chemically modified and then reduced and alkylated, was analyzed by HPLC (similarly to Figure 4): no significant differences were observed between the pH 7.9 and the pH 5 inhibitions. Thus, loss of imidate moieties via the "base" route (eq 4) did not appear to be significant under our reaction conditions.

In an effort to alter the extent of O-alkylation by generating less reactive (and more selective) carbonium ions, we tested compound **4** as an inhibitor. The 4-methoxy group should stabilize the carbonium ions formed by resonance interactions (**6**).



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Compound **4** proved to be a poor substrate for α-chymotrypsin. High substrate to enzyme ratios (of the order of 100:1) had to be used to achieve 30%–40% inhibition after 20 min, most of the substrate being destroyed by the medium (at pH 7.9, the half-life of the inhibitor in an enzyme-free system was found to be 6 min). The observation that **4** is a poor substrate for α-chymotrypsin is analogous to the low reactivity reported for the hydrolysis of L-*N*-acetyl-*O*-methyltyrosine ethyl ester by α-chymotrypsin compared to the rate for the parent tyrosine ester (Peterson et al., 1963): the hydrophobic cleft of chymotrypsin is of finite length, and elongated groups are not bound well. Note that in the case of the D family of phenylalanyl nitrosamide inhibitors (**2**), it is believed that the benzyl leaving group is bound in the aromatic pocket of the active site, whereas with simple esters of L substrates of phenylalanine, it is the phenylalanine portion that is so bound (White et al., 1977). The low extent of inhibition achieved with compound **4** prevented us from attempting an analysis of fragments possibly produced by this inhibitor: the risk of autolysis products altering the fragmentation pattern could not be neglected.

Degradation of the ^{14}C-Labeled C Chain as an Approach to the Identification of the Stable Sites of Alkylation. An average C-chain molecule from inhibited enzyme contained 0.75 benzyl group after desalting (Table II). The amino acid composition (Table I) showed no significant decrease in any residue, suggesting either that an amino acid present in high number had been alkylated (e.g., 11 Ser/C chain) or that several different residues had been partially alkylated by the benzyl carbonium ions. The amount of radioactivity associated with the C chain did not change after full hydrolysis with 6 N HCl and three lyophilizations of the hydrolyzed material, indicating that no benzyl alcohol was released (e.g., from such acid labile derivatives as *O*-benzyl-Ser or -Thr). Amino acid analysis revealed three new peaks (but of low intensity) eluting close to His; the absence of phenylalanine in the analysis indicated that the new peaks did not stem from a B-chain contaminant (the B chain has six Phe and two His, while the C chain lacks both).

Tryptic Digestion. Previous studies of tryptic digestion of C-chain derivatives of chymotrypsin showed that an insoluble "core" of residues 203–246 was readily formed (Hartley, 1964a; Maroux & Rovery, 1966); most of the residues making up the aromatic binding locus belong to the core (Blow, 1971 1976). The high reactivity and consequent low selectivity of the benzyl carbonium ions suggested that the label could well be distributed over much of the hydrophobic cleft (vide infra). Therefore, a protease hydrolysis that would solubilize the core through the formation of many small peptides (Kluh et al., 1966) would not be useful. The possibility of using chemical modification with EDC/ethylenediamine to achieve solubility of the carboxymethylated C chain and of its tryptic peptides was investigated. It has been reported that carboxymethylated proteins undergo incomplete modification (ca. 80% of the total carboxyl groups present, i.e., Asp, Glu, and CM-Cys; Frater, 1971): however, our major goal was enhanced solubility, and that was readily achieved. In a model run, the C chain from DFP-inhibited chymotrypsin was chemically modified and chromatographed on a C_{18} column; it showed two peaks, with approximate relative intensities 1 and 2, eluting at 35% and 37% CH_3CN. The amino acid composition of each peak (after desalting on Sephadex G-15) was in agreement with theory

Table IV: Amino Acid Composition of the ^{14}C-Labeled Tryptic and Chymotryptic Peptides

	T-2		T-3		T-5		CT (T-3)	
residue	found[a]	theory[b]	found[a]	theory[b]	found[a]	theory[c]	found[a]	theory[d]
CM-Cys	0.6	1	0.5	1	0	0	1.8	1
Asx	0.8	1	1.0	1	2.2	2	0	0
Thr	3.6	4	4.0	4	3.4	4	3.1	3
Ser	4.7	5	4.9	5	2.0	2	4.1	4
Glx	0	0	0	0	1.8	2	0	0
Pro	ND[e]	1	ND	1	ND	1	ND	1
Gly	3.9	4	3.8	4	1.7	1	1.9	2
Ala	2.0	2	2.3	2	4.1	4	1.0	1
Val	2.8	3	2.7	3	4.1	4	1.3	1
Met	0	0	0	0	0	0	0	0
Ile	1.0	1	1.0	1	0	0	0.7	0
Leu	1.0	1	1.3	1	2.1	2	0.1	0
Tyr	0.9	1	0.6	1	0.8	1	0.6	1
Phe	0	0	0.2	0	0	0	0	0
His	0	0	0	0	0.2	0	0	0
Lys	0.2	0	0.3	0	0.3	0	0	0
Arg	1.0	1	0.7	1	0.8	1	1.2	1
Trp	ND	2	ND	2	ND	1	ND	0

[a] Values adjusted to best fit for Asx, Glx, Gly, Ala, Leu, and Lys (whenever present). A background subtraction has been used for all amino acids (background obtained through 6 N HCl hydrolysis of HPLC eluate with no UV absorbance). [b] For peptide 204–230 (Brown & Hartley, 1966). [c] For peptide 221–245 (Brown & Hartley, 1966). [d] For peptide 216–230 (Brown & Hartley, 1966). [e] Not determined.

for the C chain (Table I). Thus, incomplete chemical modification had occurred to yield two variants of the C chain separable by HPLC. The absence of phenylalanine in the amino acid analyses (Table I) indicated the absence of contamination by the B chain.

Chemically modified DFP–C chain was then digested with trypsin, and the time course of hydrolysis was monitored by HPLC (see Figure 6 for conditions); most of the C-chain peak had disappeared after 5 min of incubation, and no major changes occurred in the tryptic map after 30 min of digestion.

Figure 6A shows the tryptic map for the DFP–C chain, monitoring at 215 nm. When the separation was repeated, but with detection set at 280 nm, the peaks eluting at 31 and 32 min disappeared, indicating that those peptides did not contain any aromatic residues. The elution order of peptides from the C chain of chymotrypsin was calculated from the composition of the expected peptides (Hartley, 1964) and coefficients for the amino acid residues (Browne et al., 1982). The peptides from the C chain of chemically modified inhibited enzyme (Figure 6B) showed the same pattern of absorption at 280 nm, and they could be shown to have the same elution order [Figure 6A and the equation of Synder et al. (1979); see also Dolan et al. (1979)]. The C chain from inhibited enzyme yielded more peptides than did the C chain from DFP enzyme, especially in the region eluting between 40 and 46 min. All of the new peaks carried some radioactive label, while no radioactivity was found in the peaks with retention times identical with those from the tryptic digest of the DFP–C chain. The difference observed in retention time between [^{14}C]benzyl peptides and "native", unlabeled peptides indicates that the presence of a benzyl group affects the retention time of a peptide on a reverse-phase column.

The recovery of radioactivity was almost quantitative (>90%). Five peaks (indicated by arrows on Figure 6B) accounted for about 60% of the total radioactivity; these major radioactive peaks were rechromatographed on the same column, eluting with a less steep gradient (from 20% to 40% CH_3CN in 30 min at 1.0 mL/min). T-4 proved to be a complex mixture, giving few radioactive peaks, while T-1, T-2, T-3, and T-5 appeared homogeneous and were analyzed for amino acid content. T-1 did not give a composition corresponding to any known sequence in the C chain, being probably a mixture of two or more peptides.

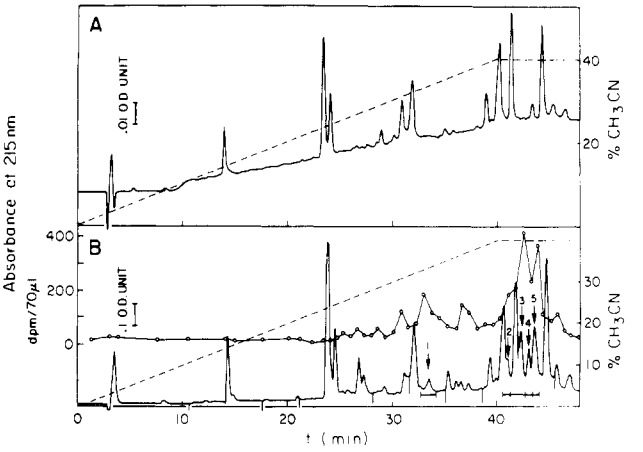

FIGURE 6: HPLC of tryptic digests. Chemically modified chymotrypsin C chain (1.0 mg/mL in 0.05 M Tris-HCl–0.02 M $CaCl_2$ at pH 8.0) was digested for 90 min at room temperature with 4% (w/w) trypsin, previously purified by HPLC on a C_{18} column (Titani et al., 1982) and used immediately. The tryptic digest was injected onto a µBondapak C_{18} column, with H_2O/CH_3CN containing 0.1% (v/v) TFA as the mobile phase at 1.0 mL/min. The dotted lines indicate the CH_3CN gradient. (Curve A) Tryptic digest of DFP C chain; (curve B) tryptic digest of the ^{14}C-labeled C chain. Fractions were collected every 0.7 min; arrows and numbers indicate major radioactive peaks. The central line (open circles) represents the radioactivity.

Results from the amino acid analyses of T-2, T-3, and T-5 are reported in Table IV. T-2 and T-3 had identical amino acid compositions, corresponding to residues 204–230 of the C chain. The presence of two peptides with identical amino acid composition but different retention times can be related to the already mentioned incomplete nature of the chemical modification of the DFP C chain. T-2 and T-3 might differ with respect to CM-Cys-220 (the only residue in the peptide carrying a carboxyl group) being coupled with ethylenediamine in one case only, or it is possible that their resolution results from a different location of the benzyl group along the chain. T-5 corresponded in composition to residues 221–245 of the C chain. The appearance of this peptide in the tryptic hydrolysate of the C chain, arising from cleavage of the Cys-220–Ser-221 bond, indicates that (carboxymethyl)cysteine coupled with ethylenediamine was recognized by trypsin as a positively charged residue, similarly to what had been re-

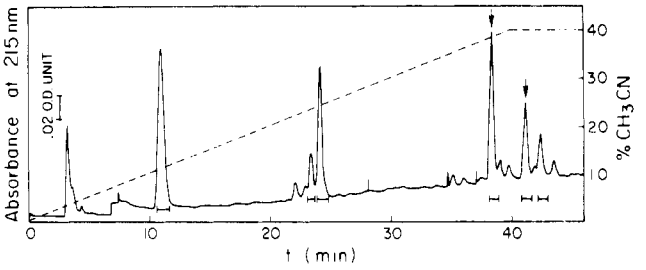

FIGURE 7: HPLC of chymotryptic digest of tryptic peptide T-3. ^{14}C-Labeled tryptic peptide 204–230 (T-3) dissolved in 1% $(NH_4)_2CO_3$ was digested for 90 min with 2% (mol/mol) α-chymotrypsin at room temperature, then lyophilized 3 times, and injected onto a μBondapak C_{18} column. Arrows indicate radioactive peaks (the peak at 41 min represents undigested T-3). Chromatographic conditions are as reported in Figure 6.

ported for (aminoethyl)cysteine (Elmore et al., 1967). The presence of a contaminating proteolytic activity in the trypsin used can be ruled out because of the HPLC purification that we performed on trypsin (Titani et al., 1982).

In order to localize more precisely in which portion of T-3 the radioactive label was present, it was digested with chymotrypsin and the products were resolved by HPLC (Figure 7). A single radioactive peak was obtained, besides some undigested starting peptide. The amino acid composition (Table IV) corresponded to residues 216–230 of the C chain, arising from chymotryptic cleavage of the Trp-215–Gly-216 bond. The lack of radioactivity in the remainder of T-3 (residues 204–215) is noteworthy.

The amino acid analyses of the tryptic and chymotryptic peptides (except for that of T-1) did not show any extra peaks, nor was there a loss of one whole residue in their amino acid compositions, observations that are similar to those made on the ^{14}C-labeled C chain, which bears 0.5–1 benzyl group/molecule. The only benzyl derivatives of amino acid residues that are stable to 6 N HCl hydrolysis are the *S*- and *N*-benzyl and aromatic *C*-benzyl derivatives. When *N*-benzyl amino acids were subjected to amino acid analysis, poor color yields (1%–10% of the parental compound) were obtained except for the Ser and Thr compounds (ca. one-third of the parental compound). Thus, with the exception of *N*-benzyl-Ser and -Thr, amino acid analysis with ninhydrin detection will not reveal the presence of new *N*-benzyl amino acids.

Conclusions

The enzyme-activated-substrate inhibition of α-chymotrypsin with *N*-nitrosamides **1** and **2** leads to a multisite alkylation of the C chain of the enzyme by the benzyl carbonium ions formed. Gdn-HCl at 6 M has been found useful as a solvent for dissolving the core peptides of chymotrypsin, and its use as an eluant on Sephadex G-75 has permitted separation of the peptides formed in the inhibition process. A chemical modification step also allowed solubilization of the peptides in dilute acid, permitting a rapid scanning of the peptides by HPLC techniques.

Consistent with model studies, alkylation of chymotrypsin occurs at labile centers (O) and at stable centers (N, S, and C); alkylation of the amide linkages of the protein occurs at both O and N. The major labile site, accounting for about 8% of the total inhibited enzyme (Table II), has been identified as the carbonyl oxygen of Ser-214 (see eq 2). A second, minor labile site has also been detected. The stable alkylation sites appear to be clustered between residues 216–230, on the basis of analysis of tryptic peptides of the C chain. Full hydrolysis of [^{14}C]-**2**-inhibited chymotrypsin followed by two-dimensional chromatography on silica gel and autoradiography revealed a minimum of three labeled amino acids, while hydrolysis of the [^{13}C]-**2**-inhibited enzyme and examination of the product by ^{13}C NMR spectroscopy at 100 MHz indicated that seven major stable alkylated amino acids had been formed. Three of these, with benzyl CH_2 resonances at 53.17, 52.21, and 51.24 ppm, are indicative of *N*-benzyl amino acids (*N*-benzylserine has a benzyl CH_2 resonance at 51.25 ppm) (J. P. Cousins, unpublished work).

To predict the most likely sites of alkylation by the carbonium ions generated in the inhibition process (eq 1), we used a molecular model of γ-chymotrypsin[4] with nitrosamide **2** fit into the active site (White et al., 1977, 1981) and also a schematic representation of the active site of α-chymotrypsin (Blow, 1976). The most likely sites for oxygen are the carbonyl groups of Cys-191, Met-192, Ser-214, Gly-216, Ser-217, Ser-218 and Thr-219 and for nitrogen the amide nitrogens of Met-192, Gly-193, Gly-216, Ser-217, and Cys-220. The oxygen site identified (Ser-214) and isolation of a peptide containing a majority of the stable sites (residues 216–230) are consistent with the prediction. Full identification and quantitation of the sites of alkylation should locate the precise site of release of the benzyl carbonium ion in the active site of α-chymotrypsin.

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