Alkyl Isocyanates as Active-Site-Specific Reagents for Serine Proteases. Identification of the Active-Site Serine as the Site of Reaction†

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ABSTRACT: The inactive [^{14}C]butylcarbamoyl derivatives of chymotrypsin and elastase have been degraded to establish which amino acid residue is involved in the reaction with alkyl isocyanates. After total hydrolysis with proteolytic enzymes both enzyme derivatives gave *O*-(butylcarbamoyl)-serine as the major amino acid derivative. [^{14}C]Butylcarbamoyl-chymotrypsin was subjected to performic acid oxidation and digestion with trypsin and subtilisin BPN, and the major radioactive peptide fractions were isolated after each treatment. The final isolation of three peptides (residues 192–199, 192–195 and 187–195) accounting for 21% of the starting radioactivity demonstrated that serine-195, the active-site serine, was the derivatized residue in the inactive chymotrypsin derivative. A small quantity of [^{14}C]butylcarbamoyl-elastase was subjected to performic acid oxidation and trypsin digestion, and the tryptic peptide containing residues 182–211 was isolated and found to account for 63% of the original radioactivity. Although this larger peptide contains four serine residues, one of these, serine-188, is the active-site serine and thus presumed to be the one derivatized in the reaction of elastase with butyl isocyanate.

Butyl isocyanate and octyl isocyanate have been shown to be active site-directed inactivators of elastase and chymotrypsin, respectively (Brown and Wold, 1971, 1973). The involvement of the active site in the inactivation reaction was demonstrated by indirect methods. However, this point is clearly essential to any meaningful interpretation of the data, and it was therefore deemed necessary to determine the specific site of attachment of the alkyl isocyanate to each enzyme.

Isocyanates are known to react with amino groups (Ozawa, 1967), sulfhydryl groups (Twu and Wold, 1973), hydroxyl groups (Adams and Baron, 1965), carboxyl groups (Naegeli and Tyabji, 1934), and imidazole groups (Staab and Benz, 1961) in model systems or in proteins. Reasonably stable derivatives are formed only in the reaction with amino groups and hydroxyl groups, and since the alkylcarbamoyl derivatives obtained in the inactivation of the proteases are stable, lysine or serine residues were strongly implicated in the reaction. This paper reports the proteolytic degradation of the inactive [^{14}C]butylcarbamoyl derivatives of chymotrypsin and elastase, the isolation of *O*-(butylcarbamoyl)serine after complete proteolysis and the isolation of radioactive peptides, the characterization of which established the active-site serine as the modified residue.

Materials and Methods

Most of the materials and assay methods have been described in the companion paper (Brown and Wold, 1973), and only those unique to the present work will be detailed here.

Preparation of [^{14}C]Butylcarbamoyl-chymotrypsin and -elastase. CHYMOTRYPSIN. Since only butyl isocyanate was available with a radioactive label, chymotrypsin was inactivated with this low-affinity reagent rather than with the high-affinity octyl isocyanate. For this reason a modification of the normal inactivation was introduced in an attempt to minimize the nonspecific incorporation of reagent which is observed during the last stages of the inactivation (Brown and Wold, 1973). Chymotrypsin (100 mg in 10 ml of 0.1 M Tris buffer, pH 7.6) was titrated with small aliquots of a solution of [^{14}C]butyl isocyanate (9.12 mg/ml in acetone) until 79% inactivation had been achieved. At this stage an excess of octyl isocyanate was added to ensure complete inactivation. The reaction product was subjected to gel filtration on Sephadex G-25 to give a fraction (a_1) which contained all the protein and 53% of the added radioactivity well separated from the remaining radioactivity (fraction a_2) (see the Results section, Figure 1). The radioactivity in a_2 could be quantitatively precipitated with $BaCl_2$, suggesting that it was $H^{14}CO_3^-$, the hydrolysis product of [^{14}C]butyl isocyanate. Fraction a_1 was the starting material for all the subsequent work. It contained 0.86 mol of butylcarbamoyl groups/mol of enzyme, and 6% of this incorporated radioactivity was subsequently found to be stable to acid hydrolysis, and is therefore presumed to represent nonspecific reaction.

ELASTASE. The elastase sample used in this work had been stored for several months and had a low specific activity. For the purpose of degradation and isolation of ^{14}C-labeled peptide the presence of inactive protein should not interfere, however. Elastase (30 mg of protein in 3 ml of 0.1 M Tris buffer, pH 7.5) was treated with 1.2 mol of [^{14}C]butyl isocyanate (9.12 mg/ml in acetone) per mol of protein to give 97% inactivation. The reaction mixture was subjected to gel filtration of Sephadex G-25. The excluded peak (a_1) contained 9.9 mg of protein and 31.6% of the radioactivity well separated from the second radioactive peak (a_2). On the basis of $BaCl_2$ precipitation, the radioactivity in a_2 again appeared to be entirely due to bicarbonate. Fraction a_1 contained 1.2 mol of butylcarbamoyl groups/mol of protein (see the Results section, Figure 2).

Degradation of Proteins and Peptides. OXIDATION WITH

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PERFORMIC ACID. The inactive derivatives of chymotrypsin and elastase were oxidized with performic acid at −10° according to the procedure described by Hirs (1967) for the oxidation of ribonuclease.

DIGESTION WITH TRYPSIN. Four milliliters of settled agarose containing 0.1 mg of active, bound trypsin per ml was washed on a sintered glass filter with water, suspended in 1% NH_4HCO_3 to a final volume of 5 ml, and equilibrated at 37° in a water bath. An appropriate amount of oxidized protein (usually 0.1–1.0 μmol) was dissolved in 2 ml of 1% NH_4HCO_3 and added to the agarose-bound trypsin. The digestion was allowed to proceed for 4–5 hr at 37° with constant stirring. To terminate the digestion, the mixture was washed on a sintered glass filter under mild suction with several volumes of water. The filtrate was then lyophilized five times with 25-ml aliquots of water.

Soluble trypsin was used to digest the insoluble oxidized elastase. In this case, the trypsin was treated with a one to one molar ratio of octyl isocyanate to remove any chymotryptic contaminant prior to its use in the digestion (Brown and Wold, 1971). A 1:10 weight ratio of treated trypsin to substrate was used and the digestion was carried out for 6 hr under the same conditions as those described above.

DIGESTION WITH SUBTILISIN. An aliquot of settled agarose (1–2 ml) containing 0.02 mg of active bound subtilisin BPN (Nagarse, Teikoku Chemical Industry Co.) per ml was washed on a sintered glass filter with water and suspended in 1% NH_4HCO_3 to a final volume of 5 ml. The peptide to be digested (usually 0.1–0.2 μmol) was dissolved in 1 ml of 1% NH_4HCO_3, added to the agarose bound Nagarse, and incubated at room temperature for 15 hr with constant stirring. The digestion was terminated by washing the digestion mixture on a sintered glass filter under mild suction with several volumes of water. The filtrate was then lyophilized five times with 25-ml aliquots of water.

PROTEIN HYDROLYSIS AND AMINO ACID ANALYSIS. Proteins and peptides were hydrolyzed at 110° for 21 hr in 6 M HCl. After removal of the HCl by evaporation on a flash evaporator, samples were analyzed on a Beckman Model 120C automatic amino acid analyzer following the procedure of Moore *et al.* (1958) as modified for accelerated analysis (Benson and Patterson, 1965). For certain samples, from which butylcarbamoylamino acid derivatives were to be recovered, the hydrolysis was carried out with proteolytic enzymes. The enzymes used in the digestion were all attached to agarose beads according to the method of Cuatrecasas *et al.* (1968). This permitted the use of high concentrations of enzyme without introducing protein contaminants. For complete hydrolysis, the butylcarbamoyl derivatives of chymotrypsin (8 mg) and elastase (1 mg) were first treated with aspergillopeptidase A (EC 3.4.4.17, acid protease from *Aspergillus saitoi* (Ichishima, 1970), generously supplied by Dr. F. Yoshida) for 18 hr at pH 1.5–2, then simultaneously with Pronase (repurified, Sigma Chemical Co; (Narahashi, 1970)) and amino peptidase M (from hog kidney, Henley and Co; (Pfleiderer, 1970)) in 0.1 M borate buffer, pH 7.6 for 16 hr, and finally in the same buffer with prolidase (EC 3.4.3.7, Miles Laboratories, Inc.) for 2 hr. All the incubations were carried out at 37° and according to past experience this procedure should give essentially complete digestion of the substrate proteins (C. C. Q. Chin, personal communication).[1]

[1] The preparation and characterization of the different agarose-protease derivatives as well as the detailed procedures for protein digestion will be described in a future publication.

An aliquot of the final digest was diluted with starting buffer and applied directly to the amino acid analyzer. When [^{14}C]-butylcarbamoylamino acids were sought, an internal standard of [^{3}H]glycine was first added, then the eluate from the analyzer long column was collected directly from the column exit, and the distribution of ^{14}C and ^{3}H was determined. The [^{3}H]glycine peak and the buffer change position served as calibration points in correlating the elution pattern obtained in this manner to the normal ninhydrin color tracing obtained in the standard runs.

Analyses of Radioactivity. The radioactivity determinations were done in a Beckman Model LS-133 liquid scintillation spectrometer. Samples were solubilized and counted in 10 ml of Beckman toluenefluoralloy (TLA) cocktail containing 10% (v/v) Bio-Solv formula BBS-3 solubilizer. The counting efficiency in this system was 97% for ^{14}C and about 40% for ^{3}H.

Results

Isolation of O-(Butylcarbamoyl)serine from Inactivated Chymotrypsin and Elastase. The first step in the characterization of the protease derivatives was an attempt to isolate and identify the derivatized amino acid residue from hydrolysates of the proteins. The two most likely derivatives, *O*-(butylcarbamoyl)serine and N^{ϵ}-(butylcarbamoyl)lysine were synthesized and characterized by Mr. C. C. Q. Chin in this laboratory. Both derivatives are stable under the conditions used in the performic acid oxidation and in the different chromatographic procedures employed in this work, but the lysine derivative is partially destroyed and the serine derivative is completely destroyed during acid hydrolysis (6 N HCl, 20 hr, 110°). *O*-(Butylcarbamoyl)serine elutes from the long column of the Spinco amino acid analyzer about 2 min after alanine, and N^{ϵ}-(butylcarbamoyl)lysine between isoleucine and leucine (C. C. Q. Chin, personal communication).

When the [^{14}C]butylcarbamoyl derivative of chymotrypsin was subjected to proteolytic digestion with the four proteases, the resulting amino acid analyses suggested that the protein had been nearly completely hydrolyzed (only the cystine recovery was significantly lower than theory (60%)), and 82% of the radioactivity in the reaction mixture was recovered in a single peak which cochromatographed with authentic *O*-(butylcarbamoyl)serine on the long column of the amino acid analyzer. No other radioactive peak was observed. In the case of the elastase derivative two major radioactive peaks were observed, one (51%) cochromatographed with *O*-(butylcarbamoyl)serine and the other (18%) with N^{ϵ}-(butylcarbamoyl)lysine. In addition traces of other radioactive peaks were observed in a region where di- and tripeptides elute, suggesting that the digestion in this case was incomplete. The presence of a significant amount of the lysine derivative was expected, because of overreaction (1.2 mol/mol incorporated) of the unexpectedly low content of active enzyme in the original reaction mixture. These data, together with the observation that essentially all of the radioactivity in both derivatives was lost during acid hydrolysis, show that the major derivative formed in the reaction of the two enzymes with butyl isocyanate involves the hydroxyl group of a serine residue. In order to establish which serine was involved in the reaction, it was necessary to degrade the inactivated enzymes and isolate and identify unique butylcarbamoyl peptides.

Isolation of [^{14}C]Butylcarbamoyl-Peptides from Chymotrypsin. The degradation procedure was very similar to that used by Shaw *et al.* (1964). The general procedure and the

TABLE I: Amino Acid Composition of Peptide Fractions b_2 and c_1 (Figure 1) from [^{14}C]Butylcarbamoyl-Chymotrypsin.

	Molar Ratio of Amino Acids[a]					
Amino Acid	Fraction b_2[b] (A)	Calcd for 0.65 mol of B Chain (B)	A − B	Calcd for 1 mol of C Chain	Fraction c_1	Calcd for Peptide 178–202
Cysteic acid	7.9	2.6	5.3	5	2.6	3
Aspartic acid	17.5	8.5	9.0	9	*2.0*	2
Threonine	15.6	8.5	7.1	9	1.0	0
Serine	17.6	9.8	7.8	11	3.6	4
Glutamic acid	9.7	4.6	5.1	4	0.2	0
Proline	6.4	1.9	4.5	4	0.9	1
Glycine	19.7	7.9	11.8	10	4.8	5
Alanine	18.4	7.2	11.2	10	3.0	3
Valine	16.3	8.5	7.8	8	2.1	2
Methionine	*2.0*	0	2.0	2	1.5	2
Isoleucine	6.6	3.9	2.7	3	0.9	1
Leucine	13.3	4.6	8.7	8	1.2	1
Tyrosine	3.0	1.3	1.7	2	0.6	0
Phenylalanine	*3.9*	3.9	0	0	0	0
Lysine	12.6	5.2	7.4	6	0.9	1
Histidine	1.3	1.3	0	0	0	0
Arginine	1.6	0.7	0.9	2	0	0

[a] The molar ratios are normalized relative to the italicized amino acids. All values are rounded off to the nearest 0.1 residue. Methionine was determined as the sulfone. Tryptophan was not determined. [b] Since B chain (residues 16–146) uniquely contains two histidines and six phenylalanines and C chain (residues 149–246) two methionines, the relative content of these three amino acids in the original hydrolysate suggested the B chain and C chain were present in the ratio 0.65:1.0.

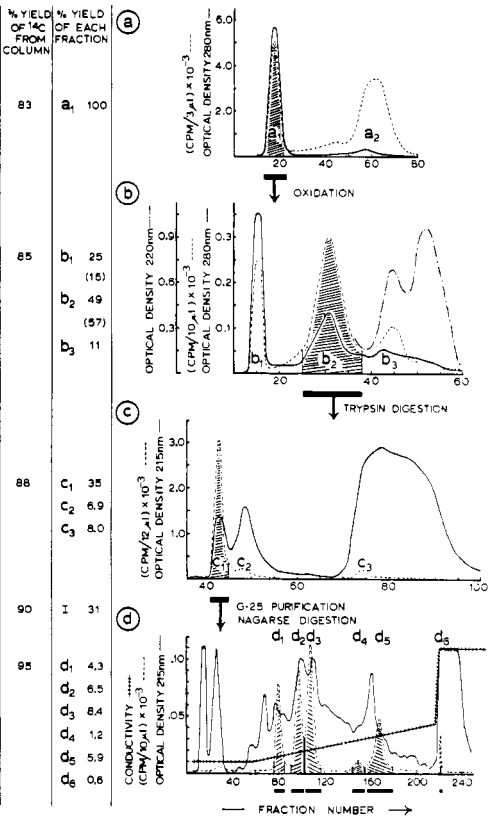

FIGURE 1: The degradation of and the isolation of radioactive peptides from [^{14}C]butylcarbamoyl-chymotrypsin. The left-hand column gives the recovery of total radioactivity in each individual step, while the second column relates the yield of radioactivity in each individual fraction to the starting material (a_1). (a) Gel filtration of the reaction mixture obtained after inactivation of chymotrypsin with [^{14}C]butyl isocyanate followed by octyl isocyanate. The reaction mixture was applied to a Sephadex G-25 (fine) column (1.7 × 110 cm) which was equilibrated and eluted with 0.1 M NH_4OAc (pH 8.5); 7.5-ml fractions were collected at a flow rate of 0.75 ml/min. Fraction a_1 was lyophilized, oxidized with performic acid and again lyophilized. (b) Gel filtration of oxidized fraction a_1. The oxidized sample was dissolved in 8 M urea and applied to a Sephadex G-75 column (2.5 × 75 cm) which was equilibrated and eluted with 0.01 M NH_4OAc; 6.8-ml fractions were collected at a flow rate of 0.68 ml/min. Fraction b_1 (25%) was collected, reoxidized, and refractionated on the same column to give two peaks corresponding to b_1 (now containing 16% of the radioactivity) and b_2 (8% of the radioactivity). The two fractions b_2 were pooled (57%) and digested with trypsin. (c) Phosphocellulose chromatography of trypsin-digested fraction b_2. The digested sample was chromatographed on a phosphocellulose column (1.7 × 100 cm) which was equilibrated with 0.025 N phosphoric acid. The column was eluted with 0.025 N phosphoric acid (through fraction 70) and then with 0.025 N phosphoric acid containing 0.5 M KCl. Fractions of 5.3 ml were collected at a flow rate of 0.53 ml/min. Fraction c_1 was desalted on a Sephadex G-25 column (1.1 × 26 cm) which was equilibrated and eluted with 0.01 M NH_4OAc; 2.5-ml fractions were collected at a flow rate of 1.25 ml/min. The radioactive fraction was lyophilized and digested with agarose-bound subtilisin BPN (Nagarse). (d) TEAE-cellulose chromatography of subtilisin (Nagarse) digested fraction c_1. The subtilisin digest was applied to a TEAE-cellulose column (0.8 × 18 cm) which was equilibrated with 0.005 M borate buffer (pH 9). The column was eluted with 0.005 M borate buffer (pH 9) (through fraction 56), then with a linear gradient to 0.5 M borate buffer (pH 9) (through fraction 220) and finally with 0.5 M borate buffer (pH 9) containing 0.5 M KCl. The flow rate throughout the elution was 0.74 ml/min and 3.7-ml fractions were collected. Each radioactive fraction was lyophilized and subjected to gel filtration on Sephadex G-10 (equilibrated and eluted with 3 × 10^{-3} M HCl) prior to hydrolysis and amino acid analysis.

results obtained at each step are given in Figure 1. The initial gel filtration of the butyl isocyanate–chymotrypsin reaction mixture (described under Materials and Methods) is shown in Figure 1a. As already mentioned, the fraction a_1 was the starting material for the degradation and the yield in subsequent steps was related to this fraction (the third column in Figure 1). Fraction a_1 was oxidized with performic acid and the oxidized sample, after lyophilization, was dissolved in 8 M urea and subjected to gel filtration on a Sephadex G-75 column according to the method of Van Hoang *et al.* (1962) (Figure 1b). Fraction b_1, which contained 25% of the total starting radioactivity and eluted at the void volume of the column, appeared to contain unresolved B chain (chymotrypsinogen residues 16–146) and C chain (chymotrypsinogen residues 149–246) as well as unoxidized protein. For this reason, fraction b_1 was once more oxidized with performic acid and rerun on the Sephadex G-75 column. The second elution was similar to that in Figure 1b with two fractions of radioactivity eluting in the position of fractions b_1 and b_2. Fraction b_1 now constituted 16% of the total starting radioactivity and the amino acid composition did not fit any discernible peptide sequence.

The pooled b_2 fractions constituted 57% of the starting

TABLE II: An Estimate of the Ratio of Methionine to ^{14}C Content in the Different Peptide Fractions Obtained after Degradation of [^{14}C]butylcarbamoyl-Chymotrypsin.

Fraction	Met/^{14}C (mol/mol)	% Yield of Radioactivity in Fraction
b_1	2.6	16.0
b_2		
c_1	2.6	35.0
c_2	3.1	7.0
c_3	2.2	8.0
b_3	2.5	11.0
	(expected ratio: 2.5[a])	(total: 77%)

[a] The expected ratio, 2.5, is based on the known content of 2 mol of methionine, 0.8 mol of acid-labile, and 0.06 mol of acid-stable [^{14}C]butylcarbamoyl groups and 0.2 mol of octylcarbamoyl groups/mol of protein; and on the assumption that the acid-labile [^{14}C]butylcarbamoyl derivatives of peptides will not be separated from the corresponding octylcarbamoyl derivatives, but will be separated from the acid-stable derivatives. The latter are presumed to be derivatives of lysine. Methionine was determined as the sulfone.

radioactivity and had an amino acid composition corresponding to a mixture of C chains and B chains in the molar ratio of 1.6:1 (Table I).

Fraction b_3 contained A chain (chymotrypsinogen residues 1–13) contaminated with smaller amount of B chain and C chain. The radioactivity associated with fraction b_3 was present in 1.1 molar ratio with the amount of C-chain contaminant.

Fraction b_2 (pooled fractions from Figure 1b) was digested with agarose-bound trypsin, and the resulting peptides were chromatographed on a phosphocellulose column monitored at 215 nm (Chin and Wold, 1972) (Figure 2c). The major radioactive peak (c_1) (35% of fraction a_1) was desalted on a Sephadex G-25 column and analyzed for amino acid composition. The results given in Table I demonstrate that fraction c_1 is peptide 178–202.

Since peptide 178–202 contains the only two methionine residues present in chymotrypsin, it became possible to assess whether the radioactivity in the various uncharacterized side fractions also was associated with the same 178–202 peptide sequence. With 0.8 mol of acid-labile butylcarbamoyl groups incorporated per mol of enzyme, and with 2 mol of methionine/mol of enzyme, a ratio of methionine to ^{14}C of 2.5 should be observed if the methionine and the ^{14}C are associated with the same fragments. The results of such a test are given in Table II, and show that within experimental errors, 77% of the original radioactivity in the starting material a_1 could be accounted for as associated with fractions containing the two methionine residues, and therefore in analogy with the results obtained with pure fraction c_1, with peptide 178–202.

In an attempt to obtain peptides small enough to permit unequivocal identification of the derivatized amino acid residue, fraction c_1 was further digested with agarose-bound subtilisin BPN (Nagarse) and the digest was chromatographed on a TEAE-cellulose column to yield six distinct radioactive fractions (Figure 1d). Each of these fractions was lyophilized

TABLE III: Corrected Amino Acid Composition[a] of Peptide Fraction d_2, d_3, and d_5 (Figure 1) from Nagarse Digestion of Tryptic Peptide c_1.

	Molar Ratio of Amino Acids[a]					
Amino Acid	Cor Fraction d_2[b]	Calcd for Peptide 192–199	Cor Fraction d_3[b]	Calcd for Peptide 192–195	Cor Fraction d_5[c]	Calcd for Peptide 187–195
Cysteic acid	0		0.1		2.2	1
Aspartic acid	1.0	1	1.2	1	2.4	1
Threonine	0		0.3		0	
Serine	0.3	1	0.5	1	3.3	3
Glutamic acid	0.2		0		0.2	
Proline	0.9	1	0		0.2	
Glycine	3.1	3	1.1	1	1.7	2
Alanine	0		0		1.2	
Valine	0		0		0.6	1
Methionine	0.5	1	0.8	1	1.2	1
Isoleucine	0		0		0	
Leucine	1.0	1	0		0	
Tyrosine	0		0		0.1	
Phenylalanine	0		0		0.6	
^{14}C	*1.0*		*1.0*	1	*1.0*	1

[a] None of the radioactive fractions obtained after the last gel filtration step (Figure 1d) were pure, as judged from the lack of coincidence of the radioactive peak and the 215-nm absorbance. The reported amino acid analyses were therefore corrected as detailed below. Normalization of each analysis was carried out as follows. A known amount of [^{14}C]peptide was hydrolyzed, and although all the radioactivity was lost during the hydrolysis, quantitative transfer and dilution techniques permitted us to determine precisely what fraction of the total peptide was applied to the amino acid analyzer column. All the amino acid molar ratios were thus related to total peptide hydrolyzed (based on ^{14}C content). All the values are rounded off to the nearest 0.1 residue. Methionine was determined as the sulfone. Tryptophan was not determined. [b] The following corrections of the observed analyses were carried out. All three fractions (d_2, d_3, and d_5) contained varying amounts of a mixture of threonine, glutamic acid, and phenylalanine in a constant ratio of 1:0.9:0.1. None of these should be present in the parent c_1 peptide, although significant amounts of both threonine and glutamate were observed (Table I). We have no explanation for the appearance of these amino acids, and they were subtracted in the stated ratio (relative to threonine) from all three analyses. In addition to this correction, the leading edge of the 215-nm peak from gel filtration was pooled to include as much of the 215-nm-absorbing peak as possible without including any of the radioactivity. This "contaminant fraction" was hydrolyzed, analyzed and its composition subtracted from that of the radioactive peak to give the results in the table. [c] After correcting for the threonine, glutamate, phenylalanine contaminants, the composition of d_5 suggested a mixture of peptides 178–182 (recognized and normalized by the isoleucine content) and 187–195 in the ratio of about 4:1. The reported analysis has been corrected for this presumed content of peptide 178–182.

TABLE IV: Corrected Amino Acid Composition of Peptide Fraction b_3 (Figure 2b) from Tryptic Digestion of [^{14}C]Butylcarbamoyl-Elastase.

Amino Acid	Molar Ratio of Amino Acid: Cor Fraction b_3[a]	Molar Ratio of Amino Acid: Ca'cd for Peptide 182–211
Cysteic acid	2.0	2
Aspartic acid	2.0	2
Threonine	0.6	1
Serine	3.7	4
Glutamic acid	2.2	2
Proline	Nd	1
Glycine	5.9	6
Alanine	0.4	1
Valine	4.3	4
Methionine	0	
Isoleucine	0	
Leucine	2.8	2
Tyrosine	0.7	1
Phenylalanine	0.7	1
Lysine	0	
Histidine	1.4	2
Arginine	0.9	1
^{14}C	0.7	1

[a] The recorded molar ratios were corrected by subtracting the sidefraction amino acid content as described in Table III, footnote *b*. The amino acids found in the peak tube from the 215-nm peak (Figure 2b) which contained no radioactivity, were subtracted from those found in the peak tube of the radioactive peak (Figure 2b) after normalizing the "contaminant fraction" to account for all the isoleucine of the radioactive peak. The remaining residues were normalized to aspartic acid = 2, to give the reported amino acid analysis. Methionine was determined as the sulfone. Tryptophan was not determined.

and subjected to gel filtration on Sephadex G-10 in preparation for amino acid analysis. All the radioactivity of fraction d_1 was lost during lyophilization, and it was concluded that this fraction contained [^{14}C]carbonate as the only radioactive component. Fractions d_4 and d_6 were found to contain insufficient quantities of amino acids to yield meaningful results, but fractions d_2, d_3, and d_5 gave reasonable amino acid analyses, which are reported in Table III. The amino acid composition of d_2 is in excellent agreement with that expected for peptide 192–199 (Hartley, 1964) of chymotrypsin, the composition of d_3 with that for peptide 192–195, and while the composition of d_5 is less convincing, it appears to best represent a peptide containing residues 187–195. In spite of some ambiguities in the analyses, we feel that the three peptides constitute convincing evidence that the radioactivity is associated with the tetrapeptide 192–195. The sequence of this peptide is Met-Gly-Asp-Ser, and since it has been established above that a serine residue was involved in the reaction, serine-195, the active-site serine, must be the residue which is modified in the activation of chymotrypsin by butyl isocyanate.

Isolation of a [^{14}C]Butylcarbamoyl-Peptide from Elastase.

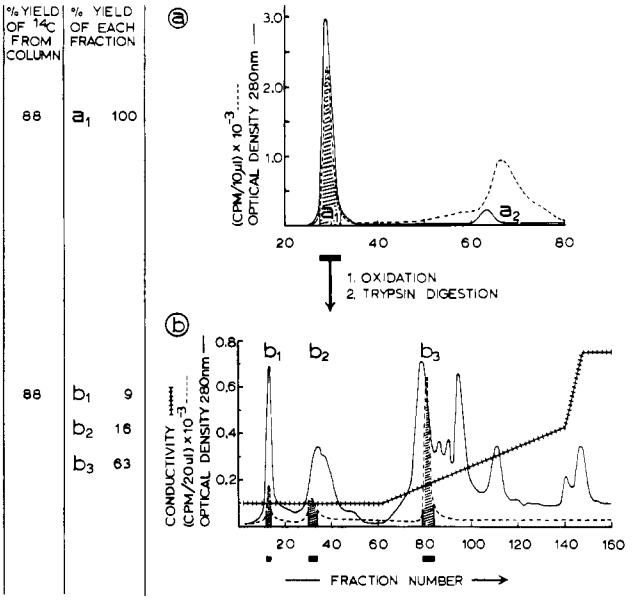

FIGURE 2: The degradation of and the isolation of a radioactive peptide from [^{14}C]butylcarbamoyl-elastase. The left-hand column gives the total recovery of radioactivity in each individual step, while the second column relates the yield of radioactivity in each individual fraction to the starting material (a_1). (a) Gel filtration of the reaction mixture obtained after inactivation of elastase with [^{14}C]butyl isocyanate. The reaction mixture was applied to a Sephadex G-25 (fine) column (1.7 × 85 cm) which was equilibrated and eluted with 0.01 M NH_4OAc; 3.4-ml fractions were collected at a flow rate of 1.7 ml/min. Fraction a_1 was lyophilized, oxidized with performic acid, and digested with trypsin. (b) Phosphocellulose chromatography of trypsin-digested fraction a_1. The digestion mixture was applied to a phosphocellulose column (1.3 × 38 cm) equilibrated with 0.025 N phosphoric acid. The column was eluted with 0.025 N phosphoric acid (through fraction 56), then with a linear gradient from 0 to 0.3 M KCl in 0.025 N phosphoric acid (through fraction 140) and finally with 0.5 M KCl in 0.025 N phosphoric acid. The flow rate was 0.5 ml/min and 5-ml fractions were collected.

Owing to the limited quantities of elastase available in this study, the characterization of elastase is less complete than that of chymotrypsin. The inactivated enzyme fraction obtained after gel filtration of the reaction mixture (a_1 in Figure 2a; see Experimental Section) was subjected to performic acid oxidation and trypsin digestion. The digestion mixture yielded three radioactive peaks (Figure 2b) and only the major one of these was analyzed. The amino acid composition of this peak (Table IV) agrees very well with that calculated for peptide 182–211 of elastase (Shotton and Hartley, 1970). Although there are four serine residues in this peptide (Ser-182, Ser-188, Ser-207, and Ser-210) and although there is no way to distinguish between these four residues on the basis of our results, we feel justified on the basis of the findings with chymotrypsin to conclude that the active-site serine (Ser-188) is the most likely site of reaction in elastase as well.

Discussion

The finding that the active-site serine was modified in the inactivation of the two proteases by alkyl isocyanate was not surprising. Based primarily on the extensive studies on chymotrypsin, the reactivity of this residue is well established. It has been modified with diisopropyl fluorophosphate (Oosterbaan *et al.*, 1958), 2-hydroxy-5-nitrotoluenesulfonic acid sulfone (Heidema and Kaiser, 1970), potassium cyanate

(Shaw *et al.*, 1964), *p*-nitrophenyl cyanate (Robillard *et al.*, 1972), 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate (Banks *et al.*, 1969), and carbamyl chloride (Erlanger and Cohen, 1963). Although with all these reagents the binding specificity is determined by different functional side groups (or in the case of cyanate by no side group at all) the reaction with chymotrypsin yields a stable derivative with serine-195 in each case. The active-site serine (serine-188) of elastase is also known to react readily with reagents that are reactive toward all the serine esterases (Naughton and Sanger, 1961; Watson *et al.*, 1970), and recently peptide chloromethyl ketone derivatives have been prepared which are related to elastase substrates and found to be specific elastase inhibitors (Powers and Tuhy, 1972). In spite of this impressive list of precedents, it was nevertheless considered important to establish unequivocally that the alkyl isocyanates indeed do react with the active-site serine. We hope to continue these studies and by crystallographic analysis determine the actual location of the alkyl side chain relative to the binding pocket in these enzymes, and the knowledge of the reaction site will be an essential prerequisite for the meaningful interpretation of such studies.

A secondary goal of this work should also be mentioned, namely, the practical application and test of some new methods. Thus, the use of insolubilized proteases for total digestion of proteins as a means of isolating and characterizing unstable amino acid derivatives was found to be feasible in this work. In fact it is unlikely that any other hydrolysis method would have permitted the isolation of *O*-(butylcarbamoyl)serine from the two enzyme derivatives in such a high yield. Another method which was evaluated in this work was a proposed general procedure for peptide separation on cellulose ion exchangers with inorganic buffers (Chin and Wold, 1972). The yield of the different peptides obtained in this study provide strong support for the general applicability of this method. In the isolation of peptides from carbamylated chymotrypsin by high-voltage electrophoresis, Shaw *et al.* (1964) obtained the tryptic peptide 178–202 in a 10% yield and two subtilisin peptides (corresponding very closely to our subtilisin peptides d_2 and d_3) in 1–2% yield. Using the phosphocellulose and TEAE-cellulose columns for the analogous purification steps, our yields were 35% for the tryptic peptide and 6–8% for each of the Nagarse peptides (total yield of 20.8%, Figure 1).

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