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Modification of a Methionine Residue near the Active Site of Chymotrypsin by *p*-Nitrophenyl Bromoacetyl-α-aminoisobutyrate*

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ABSTRACT: Reaction of chymotrypsin with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate leads to an acyl enzyme that partially reconverts to chymotrypsin at high *p*H. Intramolecular alkylation of a single methionine residue in the acyl enzyme (favored by low *p*H), followed by hydrolysis of the acyl-enzyme bond, gives an irreversibly modified chymotrypsin which possesses about 20% of the activity of chymotrypsin in a standard assay and which reacts (as does chymotrypsin) stoichiometrically with diisopropylphosphorofluoridate. The decrease in activity accompanies a 10-fold increase in the Michaelis constant over that of chymotrypsin. The modified methionine was located in the sequence -Met-Gly-Asp-Ser-Gly- at the active site of the enzyme.

The active site of an enzyme may be defined as that place possessing groups of unusual chemical reactivity at which substrate is converted to product. The efficiency of the conversion depends upon strong, rapid, and correct binding of substrate to the site and by the presence there of reactive groups oriented in the right manner to catalyze the reaction. It is convenient to break down an active site into components responsible for binding (binding site) and components involved in the catalysis per se (reaction site), and to look for constituents of both. It must be recognized, however, that close cooperation between the binding site and reaction site of an enzyme is necessary for proper function and that the two sites may have groups in common. Such cooperation should be of even more importance for an "induced-fit" mechanism (Koshland *et al.*, 1962b) than for a simple "template" mechanism (Fischer, 1894).

The identification of groups forming an active site can be pursued by both physical and chemical methods. The most direct and positive evidence for the presence of a group at the active site is its labeling, with concurrent loss of enzymic activity, by a chemical reagent. The difficulty with this approach, discussed, e.g., by Richards (1959), lies in the possibility that modification of a group some distance from the active site may for various reasons also cause inactivation. The results of chemical modification studies are most significant when they are negative (activity fully retained, proving noninvolvement of certain groups in the catalytic process), or if they are obtained by very specific reagents that rapidly label a single group or a combination of groups at the active site. The identification of serine as a component of the active site of chymotrypsin and of many other hydrolytic enzymes by specific labeling with DFP[1] (and other phosphorylating agents), and the subsequent demonstration that the sequence about this serine is similar (often -Asp-Ser-Gly-, as in chymotrypsin) in most of these enzymes, have proved the

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[1] Abbreviations used in this work: DFP, diisopropylphosphorofluoridate; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BAEE, benzoyl-L-arginine ethyl ester hydrochloride; TEE, L-tyrosine ethyl ester hydrochloride.

effectiveness of specific reagents in the study of active sites (Balls and Jansen, 1952; Cohen *et al.*, 1959).

We desired to add a new dimension to the designing of reagents for the labeling or "mapping" of active sites by taking advantage of the enzymes' specificity requirements.[2] According to this concept, the reagent has two functions, performed by at least two functional groups. The first function confers specificity for a particular active site upon the reagent, leading to chemical or physical binding at the site. The second function consists of a reactive group (alkylating, acylating, or other) that can combine chemically with a reactive amino acid side chain at or near the active site with resultant inactivation of the enzyme. Since it will not generally be known a priori what amino acid side chain will be close to the reagent at the site, the second function chosen (for preliminary work, at least) should be capable of reaction with as many types of side chains as possible. The development of such bifunctional irreversible inhibitors[3] from a generalized viewpoint is comparatively recent[2] (Baker, 1959) though earlier examples are known.[4]

In this paper we shall discuss the reaction of chymotrypsin[5] with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (compound I), which, like other nitrophenyl esters (McDonald and Balls, 1957; Oosterbaan *et al.*, 1962), specifically acylates the serine residue at the active site. The bromoacetyl group of the acyl enzyme is then attacked by a methionine residue nearby. After cleavage of the acyl-enzyme bond, the end result is an irreversibly modified chymotrypsin of decreased activity.

Experimental

Materials. α-Chymotrypsin (3× recrystallized, salt-free) and trypsin (2× crystallized, salt-free) were obtained from the Worthington Biochemical Corp. DFP, ATEE, and BAEE were purchased from Mann Research Laboratories. TEE, mp 166–167°, was obtained by Fischer esterification of L-tyrosine; the reported mp is 166° (Röhmann, 1897). *p*-Nitrophenyl pivalate (McDonald and Balls, 1957), *S*-carboxymethylhomocysteine (Armstrong and Lewis, 1951), and bromoacetyl-α-aminoisobutyric acid (Abderhalden and Haase, 1931) were prepared according to the literature. Diisopropylphosphorylchymotrypsin was prepared and crystallized twice from ammonium sulfate by the procedure of Jansen *et al.* (1949). Pivalylchymotrypsin was made as described by McDonald and Balls (1957). The C chains of performate-oxidized chymotrypsin and the modified enzyme were isolated by chromatography on Sephadex G-50 by the method of Van Hoang *et al.* (1962). All other chemicals were of the best commercial grade available.

p-Nitrophenyl Bromoacetyl-α-aminoisobutyrate (Compound I). Dicyclohexylcarbodiimide (10.3 g; 0.050 mole) was added to a solution of 11.2 g (0.050 mole) of bromoacetyl-α-aminoisobutyric acid and 7.5 g (0.054 mole) of *p*-nitrophenol in 100 ml of methylene chloride, and the mixture was stirred for 1 hour at room temperature. Dicyclohexylurea, which had formed, was filtered and washed with methylene chloride. The oil obtained by evaporation of the filtrate *in vacuo* was heated 20 minutes on a steam bath. Crystals that appeared upon cooling were recrystallized from 150 ml of ethanol to give 13.6 g of pale-yellow plates of *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (compound I), mp 149–150° (uncorr). An additional 1.2 g, mp 148–150°, was obtained from the filtrate to make the total yield 14.8 g (86%).

Anal.[6] Calcd for $C_{12}H_{13}N_2O_5Br$: C, 41.75; H, 3.80; N, 8.12. Found: C, 42.02; H, 3.80; N, 7.88.

Inactivation of Chymotrypsin by p-Nitrophenyl Bromoacetyl-α-aminoisobutyrate (Compound I). The following procedure is a typical preparation of stoichiometrically modified chymotrypsin. To a solution of 30 mg (1.2 μmoles) of chymotrypsin in 20 ml of 0.1 M acetate buffer of *p*H 5.0 was added with swirling a freshly prepared solution of 6.2 mg (18 μmoles; 15-fold molar excess) of the nitrophenyl ester I in 2 ml of ethanol. The solution was allowed to stand at room temperature for 5 hours, and was dialyzed overnight against distilled water in the cold room. The activity of the modified enzyme, as compared to that of a chymotrypsin control, was 22%.

This procedure was modified for various purposes by changing the *p*H of the buffer or ratio of nitrophenyl ester I to enzyme, or by adding reversible chymotrypsin inhibitors. In large-scale preparations (1–2 g of enzyme) all ingredients are increased proportionately and it is necessary to dialyze for 3–4 days against several changes of water to remove the nitrophenol. The modified chymotrypsin is stable for weeks in solution at 0° or in a frozen solution, and probably much longer as a lyophilized powder.

Methods. The concentration of chymotrypsin solutions of *p*H 4–5 in mg/ml was obtained by multiplying the optical density at 280 mμ by the factor 0.495 (Wu and Laskowski, 1955). The molecular weight of chymotrypsin was taken as 25,000 (cf. Desnuelle, 1960).

Chymotryptic and tryptic activities were assayed essentially according to Schwert and Takenaka (1955),

[2] The same general approach to the modification of active sites in enzymes and antibodies (reviewed by Baker, 1964) has been developed in several laboratories.

[3] The nomenclature in this field has not yet solidified (cf. the papers discussed by Baker, 1964), but we prefer this term because all the effective compounds studied to date are irreversible enzyme inhibitors, and the term "bifunctional reagent" is better applied to compounds containing two reactive functions but no particular group that would confer specificity for a given site on the reagent. Such bifunctional reagents have been used for the study of tertiary protein structure, e.g., Zahn and Steurle (1959).

[4] For example, substrate-similar anticholinesterases reviewed by O'Brien (1960) and Heath (1961), and the work on azaserine as an inhibitor of glutamine-utilizing enzymes (French *et al.*, 1963, and earlier papers).

[5] α-Chymotrypsin (E.C. 3.4.4.5).

[6] By Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

with 3 ml of 0.001 M TEE in 0.05 M phosphate buffer of *p*H 6.5, and 3 ml of 0.001 M BAEE in 0.05 M Tris buffer of *p*H 8.0, respectively, as substrates. After the addition of chymotrypsin (*ca.* 25 μg) or trypsin (*ca.* 10 μg) to the appropriate substrate, the reaction was followed at 233.5 mμ (chymotrypsin) or at 253 mμ (trypsin) versus a substrate blank in the Cary Model 14 recording spectrophotometer.

The rate of the burst of nitrophenol in the reaction of the nitrophenyl ester I (and other nitrophenyl esters) with chymotrypsin was followed at 347.5 mμ in a Cary Model 14 recording spectrophotometer. At this wavelength, an isosbestic point, *p*-nitrophenol exhibits an $\epsilon = 5500$, which is constant at least in the *p*H range from 4 to 8. In a typical experiment, 200 μl of nitrophenyl ester solution (5.5×10^{-3} M in acetone) was added to 2.8 ml of 0.1 M buffer. After observation of the spontaneous hydrolysis rate, 200 μl of chymotrypsin (*ca.* 30 mg/ml) was added and the enzymic reaction followed. If the spontaneous hydrolysis is negligible, it is simpler to add the nitrophenyl ester directly to a solution of the enzyme (2 mg/ml) in the buffer.

The incorporation of phosphorus into enzyme samples in the reaction with DFP was determined as follows: To 15 ml of an aqueous solution of native or modified chymotrypsin (25 mg, 1 μmole) were added 1 ml of 1.0 M Tris buffer of *p*H 7.5 and 1.5 ml of a solution of DFP (10 μmoles) in 2-propanol. After standing overnight, the solution was dialyzed for 4 days against several changes of 10^{-3} M hydrochloric acid, and the enzyme concentration and activity (<0.25%) were determined. Aliquots corresponding to *ca.* 0.5 μmole of enzyme were evaporated and wet-ashed in micro-Kjeldahl flasks with 1 ml of perchloric acid–nitric acid–sulfuric acid (180:65:45). The cooled solutions were neutralized with 5 N sodium hydroxide using several drops of a saturated solution of 2,4-dinitrophenol as indicator. Colors were developed by the A.O.A.C. (1945) method, read at 650 mμ on a Coleman Senior spectrophotometer, and compared with values from a freshly prepared standard curve.

Amino Acid Analyses. Samples of native or performic acid–oxidized protein (4–5 mg) (Hirs, 1956) were mixed with constant-boiling hydrochloric acid, deaerated, and hydrolyzed *in vacuo* for 22–24 hours at 105°. After evaporation of the acid, unoxidized samples were exposed to *p*H 7.5 for several hours to reoxidize cysteine. Analyses were carried out by the method of Spackman *et al.* (1958). The integration constants of these authors for the normal amino acids and of Neumann *et al.* (1962) for homoserine and its lactone and *S*-carboxymethylhomocysteine were used. An integration constant of 3.64 was determined for α-aminoisobutyric acid. Molar ratios of amino acids were calculated on the basis of leucine = 19.0. Tryptophan was estimated by a spectrophotometric method (Patchornik *et al.*, 1960).

Cyanogen bromide cleavages were done as described by Gross and Witkop (1962). Methionine peptide cleavages (Lawson *et al.*, 1962) were carried out by adjusting the *p*H of a solution of 20 mg of C chain in water to 4.0 with 1.7% triethylamine and heating the solution for 1 hour at 100°. Both heated and unheated solutions were then dinitrophenylated, after the *p*H was brought to 8.5 with 1.7% triethylamine.

Dinitrophenylated proteins were prepared as follows. A magnetically stirred mixture of 10–60 mg of protein, 3 ml of water, 2 ml of ethanol, and 100 μl of dinitrofluorobenzene was kept at *p*H 8.5 for 90 minutes by a *p*H-stat (Radiometer, Copenhagen) with 1.7% triethylamine in water as the titrant. After dilution with water the mixture was freeze-dried; the residue was extracted four times with 25 ml of absolute ether and dried.

Dinitrophenyl proteins were hydrolyzed for 8 hours at 100° in 60% perchloric acid–acetic acid (1:9) according to Hanes *et al.* (1952) to protect DNP-glycine. The ether-soluble dinitrophenylamino acids were chromatographed in the "toluene"-phosphate system and the spots were evaluated as described by Levy (1955), except that optical densities were measured in 0.1 N hydrochloric acid at 400 mμ to minimize the effect of traces of dinitrophenol, which lies close to DNP-glycine and DNP-alanine in the chromatograms.

Kinetic constants of chymotrypsin and modified chymotrypsins were obtained by the method of Cunningham and Brown (1956) with ATEE as substrate; the reactions were followed in a *p*H-stat with recorder (Radiometer, Copenhagen).

Results

Synthesis of p-Nitrophenyl Bromoacetyl-α-aminoisobutyrate (Compound I). Preliminary attempts to couple bromoacetyl-α-aminoisobutyric acid (compound II) with *p*-nitrophenol by the dicyclohexylcarbodiimide method (Bodanszky and Du Vigneaud, 1959) at room temperature or below failed. It is known that benzoyl-α-aminoisobutyric acid readily gives an azlactone that is much more stable than those derived from less highly α-substituted amino acids (Mohr and Geis, 1910; Mohr and Stroscheim, 1910). Further, azlactones are relatively unreactive toward alcohols at room temperature. For example, the half-life of 2-phenyl-5-oxazolone (derived from hippuric acid) in neutral alcohol at 25° is 16 hours (Cornforth, 1949). It seemed likely that bromoacetyl-α-aminoisobutyric acid (compound II) had been converted into its azlactone III, which did not react with *p*-nitrophenol at room temperature (Figure 1). In support of this view, a compound, presumably the azlactone III, was obtained in low yield from compound II by the acetic anhydride method (Cornforth, 1949) and smoothly converted into the nitrophenyl ester I by fusion with nitrophenol (reaction at room temperature is very slow or nonexistent). The coupling reaction using dicyclohexylcarbodiimide was then carried out successfully as described under Experimental.

Inactivation of Chymotrypsin. The reaction of chymotrypsin with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (compound I) was studied in the *p*H range 5–7. The initial burst of nitrophenol at *p*H 5 is about ten

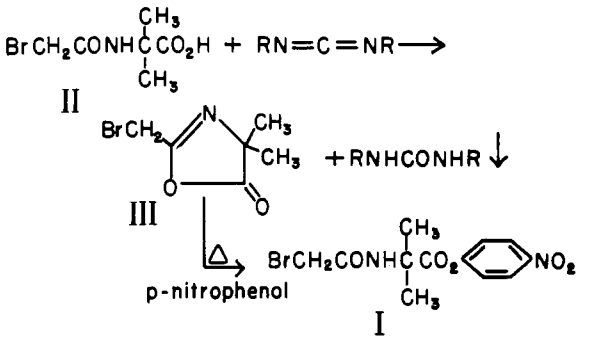

FIGURE 1: Formation of the nitrophenyl ester I. R = cyclohexyl.

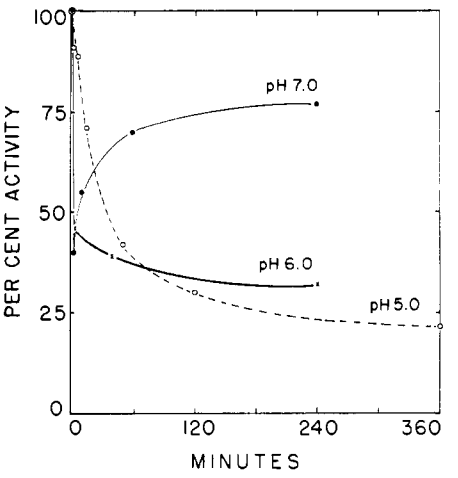

FIGURE 2: Effect of *p*H on the inactivation of chymotrypsin by the nitrophenyl ester I. The molar ratio of inhibitor to chymotrypsin was 10 at *p*H 5, 22 at *p*H 6, and 44 at *p*H 7. The enzyme concentrations in these experiments were 1.3, 2.0, and 1.0 mg/ml, respectively.

times slower than that obtained with nitrophenyl acetate. Inactivation of the enzyme by compound I occurs more rapidly the higher the *p*H. At *p*H 5 the activity drops slowly (3–4 hours) to a final value of 20–30% and remains constant; it is not reduced by further treatment with the nitrophenyl ester I. At *p*H 7, after a rapid drop in activity (<1 minute), a slow and incomplete reactivation takes place (Figure 2 and Table I).

TABLE I: Inhibition of Chymotrypsin (CT) by the Nitrophenyl Ester I at *p*H 5. Effect of 3-Indolepropionic Acid (IPA) and Bromoacetyl-α-aminoisobutyric Acid (BAIbut) on Final Activity.

CT (mg/ml)	Ratio I/CT	Added Substance (S)	Ratio S/CT	Activity[a] (%)
1.3	0			100
1.3	2.5			77
1.3	5			39
1.3	10			23
1.3	20			22
1.8	10	IPA	5	35
1.8	10	IPA	25	42
1.8	10	IPA	100	60
1.2		BAIbut	25	99
1.2		BAIbut	50	105
1.2		BAIbut	100	99

[a] Activity was determined after a 5-hour reaction time followed by overnight dialysis.

The acyl enzyme formed from compound I and chymotrypsin would be expected to be rather stable by analogy with pivalylchymotrypsin (McDonald and Balls, 1957). The latter, though stable at *p*H 5, undergoes complete hydrolysis to active chymotrypsin at *p*H 8. In contrast, the activity of chymotrypsin inactivated by compound I at *p*H 5 and allowed to dialyze overnight is unaffected by exposure to *p*H 8. Some recovery is achieved if the *p*H of the reaction mixture is brought to 8 before too much time has elapsed (<2 hours), as would be expected from the results obtained at *p*H 7. These facts are compatible with the formation of a relatively stable acyl enzyme (inactive), which either may be hydrolyzed to chymotrypsin (favored by high *p*H) or converted by alkylation to another enzyme possessing 20–30% of the activity of chymotrypsin.

Specificity of the Inhibition for the Active Site. We conclude that irreversible inactivation occurs subsequent to a reaction at the active site for the following reasons: (1) The rate of the burst of nitrophenol in the formation of the acyl enzyme is retarded (Table II) by the presence of the reversible chymotrypsin inhibitors, 3-indolepropionic acid (Neurath and Gladner,

TABLE II: Effect of 3-Indolepropionic Acid (IPA) and Hydrocinnamic Acid (HCA) on the Rate of the Burst of Nitrophenol in the Reaction of Compound I with Chymotrypsin (CT) (0.24 μmole/3 ml at *p*H 5).

Ratio I/CT	Ratio IPA/CT	Ratio HCA/CT	Relative Burst Rate[a]
4.6			100
4.6		4.6	79
4.6	4.6		87
4.6	46.		20

[a] Per cent of the rate in the absence of reversible inhibitors.

1951), and hydrocinnamic acid (Kaufman and Neurath, 1949). 3-Indolepropionic acid lowers the degree of final inhibition of the enzyme (Table I). These reversible inhibitors would be expected to compete with the nitrophenyl ester I for the active site. Since spontaneous hydrolysis of compound I is not insignificant even at *p*H 5, a portion of the enzyme is not inactivated in the presence of 3-indolepropionic acid. In contrast, reversible inhibitors afford only temporary protection of the enzyme against irreversible inhibitors that are not readily decomposed under the reaction conditions (Schramm and Lawson, 1963). (2) Bromoacetyl-α-aminoisobutyric acid (compound II), not expected to have any affinity for the active site, does not cause any inactivation at 100-fold molar excess for several hours (and probably much longer) (Table I), nor does it lower the rate or extent of inactivation by the nitrophenyl ester I. α-Bromoacetamide at 100-fold molar excess causes a loss of activity of 30–40% in 17 *days* at *p*H 5 (Schramm and Lawson, 1963). Therefore, an alkylating agent per se is not necessarily effective as an inhibitor. To be effective, it must be attracted in some way to the active site. (3) Diisopropylphosphoryl-chymotrypsin is not affected chemically (*vide infra*) by the nitrophenyl ester I, and chymotrypsinogen so treated is capable of complete activation by trypsin to chymotrypsin. These two derivatives of chymotrypsin are enzymatically inert, and therefore are incapable of reacting with compound I to form an acyl enzyme.

Intact Nature of the Hydrolytic Site in Chymotrypsin Inactivated by the Nitrophenyl Ester I. The maximum decrease in activity of chymotrypsin in the reaction with the nitrophenyl ester I is about 80%, as measured in the standard assay. The modified enzyme could represent a mixture of 20% active chymotrypsin and 80% completely inactive chymotrypsin, or an enzyme possessing about 20% chymotryptic activity. (Actually, the per cent activity of the modified enzyme is dependent on the substrate-enzyme ratio but this does not alter the reasoning presented here.) To determine which of these possibilities is correct, samples of chymotrypsin were inactivated to different degrees with the nitrophenyl ester I, and then treated with DFP. The latter reacts stoichiometrically with chymotrypsin to give a completely inactive enzyme containing 1 mole of phosphorus per mole of protein (Balls and Jansen, 1952). The phosphorus is bound as a diisopropylphosphate ester to the hydroxyl group of the serine residue at the active site (Cohen *et al.*, 1959). All of the samples (less than 0.25% activity) contained 1 mole of phosphorus per mole of enzyme (Table III). This indicates that the proportion of modified enzyme molecules capable of reaction at the active site is close to unity and that the activity of 20% obtained in the standard assay is a property of the modified enzyme.

Modification of Methionine. Amino acid analyses of chymotrypsin before and after inactivation by the nitrophenyl ester I (20% molar excess, 4 hours; dialysis) are given in Table IV. The results (column d) obtained with commercial α-chymotrypsin agree quite well with values (columns a, b, and c) deduced from published analyses of chymotrypsinogen, which vary somewhat among themselves (Wilcox *et al.*, 1957; Rovery *et al.*, 1960; Zmrhal, 1962). The only significant changes in the amino acid composition subsequent to modification (column e) are a decrease of the methionine content from two residues to one, the presence of one residue of α-aminoisobutyric acid, and the appearance of homoserine, homoserine lactone, and *S*-carboxymethylhomocysteine. The latter three compounds are known to be formed on hydrolysis of methionine peptides alkylated with iodoacetic acid (Gundlach *et al.*, 1959; Neumann *et al.*, 1962), and are to be expected in hydrolysates of methionine peptides alkylated with amides of bromoacetic acid (Schramm and Lawson, 1963). In the special case of inhibitor I, α-aminoisobutyric acid is expected as well. No other change in amino acid composition has been detected.

TABLE III: Phosphorus Content of Inhibited Chymotrypsins (CT) after Reaction with DFP.

Ratio I/CT	Activity[a] (%)	Ratio P/CT
0	100	1.04
5	39	1.05
10	23	1.04
20	22	1.03

[a] After inhibition with compound I and before treatment with DFP.

Amino acid analysis of the modified chymotrypsin after performic acid oxidation (column f) gives the same picture as analyses without prior oxidation. In the run given in the table, cystine was incompletely oxidized to cysteic acid but a control showed that methionine was quantitatively converted to methionine sulfone in chymotrypsin itself. Methionine sulfonium salts are stable to the oxidation. This procedure has the advantage that the quantities of α-aminoisobutyric acid, which falls just before cystine on chromatograms, may be calculated, and methionine regenerated on hydrolysis of the sulfonium salt (*ca.* 0.2 mole) may be estimated (Schramm and Lawson, 1963). The analysis of diisopropylphosphorylchymotrypsin (column g), after exposure to compound I in 25% molar excess for 6 hours followed by overnight dialysis, showed only traces of *S*-carboxymethylhomocysteine and α-aminoisobutyric acid, undoubtedly formed from methionine modified in the trace of chymotrypsin (*ca.* 0.25%) present in the phosphorylated enzyme. The methionine content was not distinguishable from that of chymotrypsin. These facts establish the rapid modification of one of the two methionine residues in chymotrypsin by the nitrophenyl ester I.

Location of the Modified Residue near the Active Site. The two methionine residues in chymotrypsin are located comparatively close together in the

TABLE IV: Amino Acid Composition of Chymotrypsin (CT); Chymotrypsin Modified by Compound I and Diisopropylphosphoryl (DIP); Chymotrypsin Treated with Compound I.[a]

Amino	CT				Modified-CT		DIP-CT
Acid	a	b	c	d	e	f	g
$CysO_2$						7.9	
Asp	20.8	20.9	21.3	21.4	21.4	22.4	21.2
$MetO_2$						1.0	
Thr	22.0	22.1	21.0	21.8	21.8	21.3	21.9
Ser	29.1	26.1	28.1	27.2	27.8	27.4	27.6
HS					0.1	0.1	
Glu	14.2	14.4	14.9	15.3	15.5	15.4	15.0
Pro	8.7	9.2	9.1	9.0	9.1	8.9	9.4
SCM					0.5	0.4	<0.01
Gly	23.3	22.7	22.2	23.4	23.6	23.1	23.0
Ala	21.7	21.6	22.0	22.5	22.6	22.2	22.4
Ibut					1.0	1.2	Trace
Cys/2	10.0	10.	9.8	9.4	9.5	1.4	5.6
Val	22.4	23.1	22.0	21.9	21.7	21.8	21.5
Met	1.9	2.0	1.8	2.1	1.2	0.2	2.1
Ileu	9.9	9.7	9.8	9.9	9.7	9.3	9.4
Leu	18.8	18.7	19.2	19.0	19.0	19.0	19.0
Tyr	4.1	4.0	4.2	4.0	3.8	3.6	4.0
Phe	6.5	6.0	6.2	6.1	6.1	6.0	6.8
Lys	13.2	13.6	13.4	13.4	12.9	14.4	12.5
His	1.9	1.9	1.9	1.7	1.8	1.4	1.5
HSL					0.3		
Arg	3.0	2.9	2.9	2.7	3.0	3.1	2.8
Try	7.0	8.	6.8	8.0	8.0		

[a] The results in columns a, b, and c, obtained with chymotrypsinogen, are decreased by one residue each of Asp, Ser, Thr, and Arg to correspond to the composition of α-chymotrypsin: (a) Wilcox *et al.* (1957); (b) Rovery *et al.* (1960); (c) Zmrhal (1962). Columns d–g are results of the present study. Columns d and e are the averages of three determinations, except for Try, which was determined once by a spectrophotometric method (Patchornik *et al.*, 1960). Column f was a single analysis after performate oxidation by the method of Hirs (1956); HSL was obscured by the ammonia peak. Column g was a single analysis. Cystine was low because the hydrolysate was not air-oxidized before analysis. Abbreviations for unusual amino acids: HS, homoserine; SCM, *S*-carboxymethylhomocysteine; Ibut, α-aminoisobutyric acid; HSL, homoserine lactone.

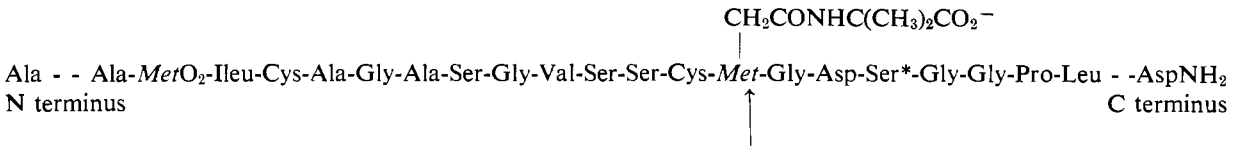

FIGURE 3: Sequence of the C chain of performate-oxidized chymotrypsin near the active site (after Hartley, 1964). Location of the modified methionine residue at position 3 from the serine (Ser*) at the active site. The arrow shows the site of the cleavage on heating.

C chain in a sequence (Figure 3) elucidated by Hartley (1964). One of them, Met-192, is the third residue from the serine (asterisk) at the active site and is attached C-terminally to glycine. The other, Met-180, is the fifteenth residue from the serine, and is bound C-terminally to isoleucine.

The methionine peptide cleavage reaction seemed to be an ideal method to locate the modified methionine residue, and could be applied in two ways. Cyanogen bromide cleavage at room temperature (Gross and Witkop, 1962) at the *unmodified* methionine should lead to the formation of a new end group (isoleucine or glycine), and simple heating (Lawson *et al.*, 1962) should cause cleavage at the methionine *modified* by compound I to give a new N-terminal group (glycine or isoleucine). Application of the cyanogen bromide

cleavage to chymotrypsin and modified chymotrypsin gave results difficult to interpret because of the numerous end groups in the starting material (commercial chymotrypsin). When twice-crystallized diisopropylphosphorylchymotrypsin, which is free of extraneous end groups (Rovery *et al.*, 1953), was subjected to the cyanogen bromide reaction, dinitrophenylation revealed new N-terminal glycine and isoleucine, corresponding to at least 40% cleavage at each methionine residue (Table V). The method was not applied to the diisopropylphosphoryl derivative of modified chymotrypsin, since we have not so far succeeded in crystallizing it.

TABLE V: Methionine Peptide Cleavage of Chymotrypsin (CT) Derivatives

Substance	Cleavage	End Groups by Dinitrophenylation (Ala = 1.00) Ileu	Gly
DIP-CT[a]	CNBr	1.82	0.46
DIP-CT[a]	None	1.42	0.00
C chain, modified CT[b]	Heated	0.04	0.37
C chain, modified CT[b]	Not heated	0.04	0.06
C chain, CT[c]	Heated	0.00	0.06
C chain, CT[c]	Not heated	0.00	0.03

[a] Twice-crystallized diisopropylphosphorylchymotrypsin. [b] C chain from modified, performate-oxidized chymotrypsin. [c] C chain from performate-oxidized chymotrypsin. This preparation was purer than (*b*), indicated by the complete absence of the isoleucine end group.

A satisfactory derivative for cleavage by heating was found in the C chain of performate-oxidized chymotrypsin, prepared by the method of Van Hoang *et al.* (1962). The amino acid composition of the oxidized C chains from chymotrypsin and chymotrypsin modified by compound I was found to be as reported by these authors. Decomposition products from the one alkylated methionine residue were, of course, present in the hydrolysate. Very little contamination (0–4%) by the B chain, as measured by the ratios of N-terminal Ileu/Ala, was observed, and other end groups were present only as traces. Dinitrophenylation of the oxidized C chain of chymotrypsin before and after heating at *p*H 4 and 100° for 1 hour showed no appreciable change in end groups. Heating of the C chain from oxidized-modified chymotrypsin, in contrast, led to an increase of 0.3 mole in N-terminal glycine, but none in isoleucine (Table V). This establishes that the methionine residue modified by the nitrophenyl ester I is the one (Met-192) closest to the serine residue of the active site in the sequence -Met-Gly-Asp-Ser*-Gly-.

Kinetic Properties of the Modified Enzyme. Michaelis constants (K_m) and kinetic constants (k_3) of chymotrypsin and the modified enzyme, determined at *p*H 8 (20° and 30°) with ATEE as substrate, are given in Table VI along with values for chymotrypsin obtained by Cunningham and Brown (1956) at *p*H 8 and 25°. The values of K_m are in good agreement with that of Cunningham, and his value for k_3 is as expected, intermediate between our values obtained at lower and higher temperatures. The K_m of the modified enzyme is increased 10-fold or more and its k_3 about 1.5-fold over those of chymotrypsin. A consequence of the increased K_m is that the per cent activity of the modified enzyme (compared to chymotrypsin) is extremely dependent upon the substrate concentration (Table VII).

TABLE VI: Kinetic Constants[a] of Chymotrypsin (CT) and Modified Chymotrypsin at *p*H 8.

	20°		25°[b]		30°	
	$K_m \times 10^2$	k_3	$K_m \times 10^2$	k_3	$K_m \times 10^2$	k_3
CT	0.06	2.5	0.07	2.70	0.07	3.9
Mod-CT	0.7	3.6			0.7	5.6

[a] Units: K_m = moles liter^{-1}; k_3 = moles liter^{-1} min^{-1} mgN^{-1} ml. [b] Cunningham and Brown (1956).

TABLE VII: Dependence of Per Cent of Activity of Modified Chymotrypsin (CT) on Substrate (ATEE) Concentration.[a]

Concn ATEE $\times 10^3$	V (initial) $\times 10^4$ (moles liter^{-1} min^{-1}) CT	Mod-CT	Activity of Mod-CT (%)
7.50	6.49	5.37	83
3.75	5.79	3.77	65
2.25	5.45	2.53	46
1.5	4.75	1.64	35
0.75	3.65	0.92	25

[a] Data taken from a K_m determination run at 20°.

Representative values of the kinetic constants determined at different *p*H values and 20° are given in Table VIII. They were taken from another series of measurements than those in Table VI, and the slight differences in the values for *p*H 8 and 20° reflect the experimental errors involved. From these and similar data on the variation of k_3 with *p*H, the pK_a of the modified enzyme was calculated by the method of Cunningham and Brown (1956) to be 6.7 at 20° in its reaction with ATEE. The variation in behavior of the

modified chymotrypsin at different *p*H values is thus the same as that of chymotrypsin, for which a pK_a of 6.74 versus ATEE at 25° was determined (Cunningham and Brown, 1956). These authors also found that the K_m of chymotrypsin was constant except at high *p*H values, when it increased. We have found this to be characteristic of both modified and unmodified chymotrypsins.

TABLE VIII: *p*H Dependence of the Kinetic Constants[a] of Chymotrypsin (CT) and Modified Chymotrypsin at 20°.

	Chymotrypsin		Modified Chymotrypsin		Ratio k_3 (CT)/ k_3 (Mod-CT)
*p*H	$K_m \times 10^2$	k_3	$K_m \times 10^2$	k_3	
6.0	0.052	0.41	1.1	0.74	1.8
7.0	0.060	1.5	0.82	3.0	2.0
8.0	0.051	2.2	0.88	3.7	1.7
8.5	0.086	2.3	1.1	4.4	1.9
9.0	0.10	2.2	1.3	4.3	2.0

[a] Units are as in Table VI.

Reaction of the Nitrophenyl Ester I with Trypsin.[7] Trypsin also catalyzes the hydrolysis of the nitrophenyl ester I. Attempts to inhibit trypsin under the conditions more vigorous than those described under Experimental for chymotrypsin (with the incorporation of calcium chloride, 0.05 M, in the buffers) at *p*H 5 and 7 led to only a slight reduction in activity, comparable to that observed with untreated trypsin blanks and undoubtedly due to autolysis. No change in amino acid composition was observed.

Discussion

A bifunctional irreversible inhibitor owes its reactivity toward a component of an active site primarily to prior binding there, achieved by its possession of some feature or features common to substrates. The enhancement in reactivity of such an inhibitor toward a reactive group at the active site, in contrast to its reactivity toward similar groups elsewhere on the protein, is due to its concentration at the site (see Wofsy *et al.*, 1962). This results in a virtually intramolecular reaction between the inhibitor and the reactive group if the initial binding is physical only. Examples are the inactivation of chymotrypsin by a chloroketone derived from *p*-toluenesulfonyl-L-phenylalanine, with concomitant modification of the histidine residue linked C-terminally to cystine (Schoellmann and Shaw, 1963; Ong *et al.*, 1964), and the inhibition of chymotrypsin by α-bromoacetanilide and similar compounds, accompanied by the alkylation of a single methionine residue (Schramm and Lawson, 1963). With a second type of inhibitor, exemplified by the nitrophenyl ester I, the initial absorption step is followed by chemical combination with the active site, analogous to the combination of enzyme and substrate. Chemically fixed in place at the active site, the reactive group on the inhibitor can react intramolecularly with an amino acid side chain nearby.

The reaction of *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (compound I) with chymotrypsin is specific for the active site, since reversible inhibitors retard the reaction and nonspecific alkylating agents do not affect the enzyme for long periods of time. The primary site of attack, by analogy with the reaction of *p*-nitrophenyl acetate, is the serine residue at the active site (Oosterbaan *et al.*, 1962). Acylation of the serine hydroxyl group is expected to be preceded by rapid reversible binding at the specificity site; the K_m in the acylation step of the reaction of *p*-nitrophenyl acetate with chymotrypsin is 1.12×10^{-3} (1.4% acetonitrile, *p*H 7.8; Kèzdy and Bender, 1962), which is not much greater than the K_m of 0.7×10^{-3} (*p*H 8.0; Cunningham and Brown, 1956) obtained with a typical substrate, ATEE. The reversible inhibition of chymotrypsin by benzene and indole derivatives, both simple and complex, is well known. In the case of nitrophenyl esters the binding can be attributed to the presence of the nitrophenyl group.

Acylation with the nitrophenyl ester I leads to bromoacetyl-α-aminoisobutyrylchymotrypsin, with the loss of *p*-nitrophenol. At this point, the acyl enzyme can simply hydrolyze to give chymotrypsin and bromoacetyl-α-aminoisobutyric acid (compound II) (favored by high *p*H), or it can undergo intramolecular alkylation at the methionine residue closest to the active serine residue, followed by deacylation to give the modified enzyme. A second route to the modified enzyme, deacylation followed by alkylation, is possible. However, we favor the former explanation since (a) the acyl enzyme should be fairly stable by analogy with pivalylchymotrypsin (McDonald and Balls, 1957), and (b) several other less highly substituted nitrophenyl esters of the same general type as compound I are rapidly hydrolyzed by chymotrypsin but do not inhibit it.[8] A third conceivable route to the modified enzyme, alkylation followed by acylation and deacylation, is unlikely because the reaction at *p*H 6 and 7 proceeds in two phases (Figure 2). In the first phase the activity falls very rapidly. Subsequently, at *p*H 6 the activity continues to drop at a slow rate, while at *p*H 7 it rises fairly rapidly and levels off at a value less than 100% (77% in the experiment in Figure 2). This behavior supports the conclusion that an acyl enzyme is formed before irreversible inactivation takes place. Between 5 and 10 moles of nitrophenyl ester I are required at *p*H 5 to effect complete conversion to the

[7] We are indebted to M. David Leafer for these experiments.

[8] M. David Leafer, to be published.

modified enzyme (Table I). The average number of turnovers (hydrolyses of the acyl enzyme) before modification has not been determined, but could easily be less than five, since spontaneous hydrolysis destroys some of the nitrophenyl ester. The inhibitor is therefore rather efficient in its modification of chymotrypsin.

The reaction leading to inhibition is stoichiometric and quite specific, since the nitrophenyl ester I labels a single methionine residue in the enzyme. No other group in chymotrypsin is modified. The reactive methionine residue (Met-192, Figure 3) is separated by only two amino acids from the active serine residue at the active site. The same methionine[9] is also alkylated specifically by α-bromoacetanilide (Schramm and Lawson, 1963) and is more easily oxidized to the sulfoxide than the other methionine residue in the enzyme (Koshland *et al.*, 1962a; Schachter and Dixon, 1964). It is probable that the methionine residue alkylated by iodoacetylphenylalanine esters (Gundlach and Turba, 1962) is also Met-192. Modification of this methionine residue gives enzyme derivatives with considerably lower activity than chymotrypsin itself. Chymotrypsin oxidized at Met-192 retains the capacity to react completely with cinnamoylimidazole (Koshland *et al.*, 1962a), and chymotrypsins modified at this methionine by the nitrophenyl ester I and by the inhibitors described by Schramm and Lawson (1963) incorporate phosphorus stoichiometrically in their reactions with DFP. The decrease in activity can be accounted for by the large increase in the Michaelis constant (about 10-fold in the case of our nitrophenyl ester–modified chymotrypsin); only small changes in the maximum velocity have occurred. The reaction site is therefore unimpaired. Its decreased efficiency in comparison to that of chymotrypsin can be attributed to a decreased capacity to bind substrate. The methionine residue can be ruled out as a constituent of the reaction site, since its nucleophilic capacity as a sulfide is abolished upon alkylation to give a ternary sulfonium salt. It is tempting to speculate that Met-192 plays a role in the binding site, especially since it is so near the active serine residue. It is quite possible, however, that this methionine residue is not part of the binding site per se. Substituents on the sulfur atom of Met-192 might also interfere with substrate binding by blocking the approach to the binding site or by causing a slight change in the tertiary structure of the enzyme near the active site.

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[9] H. J. Schramm and W. B. Lawson, unpublished experiments.

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The Sulfatase of Ox Liver. IX. The Polymerization of Sulfatase A*

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ABSTRACT: Archibald and equilibrium ultracentrifugation experiments have been used to show that sulfatase A exists completely as monomer (mw 107,000) at *p*H 7.5 and as a tetramer (mw 411,000) at *p*H 5.0 in 0.10 ionic strength buffers in the protein concentration range 0.005–0.5 g/100 ml. The identical partial specific volume and optical rotatory dispersion of the protein at these two *p*H values suggest that no marked shape change accompanies the polymerization. Sephadex chromatography experiments revealed that the tetramer dissociated in the concentration range 0.3×10^{-4}–0.3×10^{-2} g/100 ml at *p*H 5.0, whereas the monomer at *p*H 7.5 remained unaltered at these concentrations. Weight-average sedimentation coefficients varied smoothly with *p*H between *p*H 5.0 and 7.5, and were concentration dependent at *p*H 6.3, the intermediate value selected for detailed study.

The results suggest that at *p*H 6.3 in the concentration range 0.1–1.0 g/100 ml a relatively stable dimer coexists in rapid equilibrium with monomer, trimer, and tetramer. A stable dimer is also formed at *p*H 7.5 if the ionic strength is increased to 2.0, suggesting that the net charge on the protein at *p*H 7.5 prevents polymerization in low ionic strength buffers. The results suggest experimental conditions for future kinetic studies which would avoid complications inherent in the study of mixtures of polymeric species. Finally, the amino acid composition of the enzyme is presented and its high proline content is correlated with its unusual optical rotatory dispersion.

In a previous communication (Nichol and Roy, 1964) a method was given for the preparation of sulfatase A (an arylsulfate sulfohydrolase, EC 3.1.6.1) from ox liver. Sedimentation velocity patterns obtained with the enzyme in 0.10 ionic strength buffers of *p*H 7.5 and 5.0 were subjected to two boundary analyses, those of Baldwin (1959) and of Fujita (1956), and by each method the material was demonstrated to be homogeneous with respect to sedimentation coefficient. However, the *s* values varied from 6 at *p*H 7.5 to 14 at *p*H 5.0, a result tentatively attributed to the polymerization of the enzyme at *p*H 5.0. The hypothesis has been tested

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