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INACTIVATION OF α-CHYMOTRYPSIN BY A BIFUNCTIONAL REAGENT, 2-BROMOMETHYL-3,1-BENZOXAZIN-4-ONE

ROBERT ALAZARD*, JEAN-JACQUES BÉCHET, ALAIN DUPAIX AND JEANNINE YON
Laboratoire d'Enzymologie Physico-chimique et Moléculaire, Groupe de Recherches du C.N.R.S. associé à l'Université de Paris-Sud, Orsay 91 (France)

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SUMMARY

α-Chymotrypsin is selectively and irreversibly inactivated by 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib) at neutral pH. Reaction of the enzyme with this activated ester leads rapidly to a relatively stable acyl-enzyme in which intramolecular alkylation of a single methionine residue (likely methionine-192) followed by hydrolysis of the acyl-enzyme bond occurs. Kinetic evidence for such a process is found in the measurements of proflavin displacement accompanying the reaction. The irreversibly modified enzyme still possesses its intact active site but its activity towards specific substrates is altered. Alkylation of the enzyme increases mainly K_m but does not change appreciably k_{cat}. The inhibition constants of specific inhibitors such as proflavin or indole are increased several times. From crystallographic data it is known that methionine-192 forms the lid of the specificity cavity in the enzyme. Therefore a bulky substituent such as the 2-acetamido benzoic acid on the sulfur atom of this methionine might sterically hinder the substrate binding by blocking the approach of the binding site.

INTRODUCTION

Bifunctional reagents have often been employed to covalently label the active sites of enzymes[1] and to establish the spatial relationship among amino acids at the active site. Such bifunctional labeling reagents have been applied particularly to the study of the active center of α-chymotrypsin[2,3], trypsin[4], sulfhydryl proteases[5,6] and aspartate aminotransferase[7].

We have studied the reactivity of a new potential bifunctional reagent, 2-

Abbreviations: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Ac-Trp-OEt, *N*-acetyl-L-tryptophan ethyl ester; Bz-Arg-OEt, *N*-α-benzoyl-L-arginine ethyl ester hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Present address: Département de Biologie, Service de Biochimie, Bat. 142, C.E.N. Saclay B.P. No. 2, Gif-sur-Yvette 91, France.

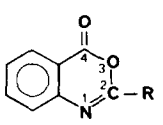
bromomethyl-3,1-benzoxazin-4-one (Compd Ib in Fig. 1), against "serine proteases" and particularly α-chymotrypsin. This compound appears very rapidly, in aqueous solution, during the hydrolysis of *p*-nitrophenyl *N*-(bromoacetyl) anthranilate (Compd III) (see ref. 8), and may be isolated at neutral pH from the reaction medium. It has been assumed that this benzoxazone Ib could behave as a pseudo-substrate in the presence of α-chymotrypsin, due to its activated ester bond. After acylation of the enzymically active serine and formation of the acyl-enzyme, the bromomethyl group would then have the opportunity to react with some other amino acid residue in its proximity. Thus Lawson and Schramm[2] have previously shown that *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (Compd IV) modifies α-chymotrypsin by intramolecular alkylation of the methionine-192 in the transiently formed acyl-enzyme. An analogous result has been found in this work and will be discussed.

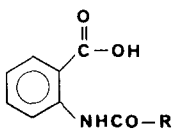
EXPERIMENTAL PROCEDURE

Materials

α-Chymotrypsin was a salt-free, three-times crystallized product (batch No. CDI 61738) purchased from Worthington Biochemical Corp. Trypsin was a crystallized product from the Novo Industri, Copenhagen (batch No. 90212).

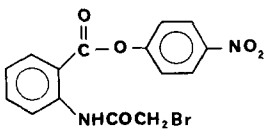
Compds Ia, Ib, IIb and III (Fig. 1) have been synthesized and characterized as previously described[8].



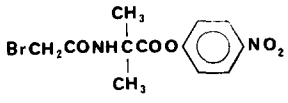


Ia R = CH_3 IIa R = CH_3

Ib R = CH_2Br IIb R = CH_2Br



III



IV

Fig. 1. Structure of compounds.

p-Nitrophenyl *N*-(bromo[2-^{14}C]acetyl) anthranilate was prepared by the dicyclohexylcarbodiimide method as described for the unlabeled analogous Compd III (see ref. 8). At the beginning of the reaction, bromo [2-^{14}C]acetic acid (50 μCi; spec.

act. 52 Ci/mole), purchased from the Radiochemical Centre, Amersham (England), was dissolved in tetrahydrofuran and was added to a mixture of bromoacetic acid (3 mM) and *p*-nitrophenyl ester of anthranilic acid (3 mM) in the same solvent. The product had m.p. 152.5–158 °C (decomposition) and exhibited the same spectral properties as the unlabeled compound. Its specific radioactivity was determined by relating the ^{14}C content to the *p*-nitrophenolate ion absorption in 0.1 M NaOH (ε at 400 nm = 18 300 l. mole^{-1}) and was found equal to about 13.5 mCi/mole.

N-Acetylanthranilic acid (Compd IIa) was synthesized following the procedure of Steiger[9], by the action of bromoacetyl bromide upon anthranilic acid, in aqueous solution, in the presence of sodium hydroxide and bromothymol blue. After decoloration of the solution with Norit and addition of ethanol (to prevent the precipitation of the sodium salt), hydrochloric acid was added to the mixture and the product precipitated after cooling. It had m.p. 185–186.5 °C (ref. 10; m.p. 184–185 °C). The ultraviolet spectrum (in dioxan) showed λ_{max} at 312 nm and 252 nm as previously reported[10].

N-Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt) were obtained from Cyclo Chemical Corp. *N*-Acetyl-L-tryptophan ethyl ester (Ac-Trp-OEt) and proflavin hydrochloride were purchased from Mann Research Laboratories. Benzamide and indole were obtained from Fluka AG Chemische Fabrik and Société Prolabo (France), respectively; α-bromo-*p*-nitroacetophenone was a product from K and K laboratories. These different compounds were reagent grade and were used without further purification.

p-Nitrophenyl acetate (Eastmann Kodak) was recrystallized from ethanol. It had m.p. 76–77 °C (ref. 11; m.p. 77.3–77.9 °C).

Dioxan and acetonitrile were purified according to Fieser[12] and Coetzee[13], respectively.

All other chemicals, including *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 2-(*N*-morpholino)ethanesulfonic acid (MES) purchased from Calbiochem, were reagent grade and were used without further purification.

Methods

Kinetic measurements. Stock solutions of native α-chymotrypsin were prepared by dissolving the crystals in 0.001 M HCl at 4 °C. Protein concentration was estimated at 280 nm, with a Cary model 14 recording spectrophotometer, using a molar extinction coefficient of $5 \cdot 10^4$ (ref. 14) and a molecular weight of $2.5 \cdot 10^4$ (ref. 15).

The absolute concentration of active sites was obtained by measuring at 400 nm the burst of *p*-nitrophenol in the enzymatic hydrolysis of *p*-nitrophenyl acetate[16], with a Cary model 16 spectrophotometer equipped with a thermostatically controlled cell compartment and coupled to a Sefram graphispot enregistror. The experimental conditions were: substrate concentration 0.57 mM; enzyme concentration 4–10 μM; 0.025 M HEPES–NaOH buffer, pH 7.6; ionic strength 0.5 (NaCl); 4.6% (v/v) acetonitrile. The molar extinction coefficient of *p*-nitrophenolate ion was 18 300 at 400 nm and the pK_a of *p*-nitrophenol was equal to 7.03 at 25 °C under our experimental conditions.

The reaction of α-chymotrypsin with 3,1-benzoxazin-4-ones Ia and Ib was followed indirectly by the proflavin displacement method[17]. The usual procedure involved the addition of 25 μl of substrate stock solution in acetonitrile to a measured

volume (2.7 ml) of enzyme–proflavin mixture. The reference cell contained the same enzyme–proflavin mixture to which were added 25 μl of solvent. The medium was buffered with 0.025 M HEPES–NaOH buffer (0.5 M NaCl).

The α-chymotrypsin-catalyzed hydrolyses of 3,1-benzoxazin-4-ones Ia and Ib were also tentatively measured spectrophotometrically at 315 nm. At this wavelength where the protein does scarcely absorb, the molar extinction coefficients of Compds Ia, IIa, Ib and IIb in aqueous solution (pH 7.0) were 2600, 450, 2750 and 900, respectively. As the hydrolysis rates in the presence of the enzyme were very slow, results were not accurate and this method was discontinued.

The enzymatic hydrolyses of ethyl esters (Ac-Tyr-OEt, Ac-Trp-OEt, Bz-Arg-OEt) were followed potentiometrically at constant pH and 25 °C, using a Vibron pH-meter model 33 B (EIL). The medium was unbuffered (0.1 M NaCl), but the pH was kept constant by addition of small known volumes of 0.1 M (or 0.05 M) NaOH with an Agla microsyringe.

The kinetic constants k_{cat} and K_m were determined from Eadie plots and a statistical analysis of these plots was performed using a Wang model 373 electronic calculator.

Measurements of the rates of deacylation of acyl-chymotrypsins in the presence of Ac-Tyr-OEt. In some cases, the deacylation run of a relatively stable acyl-enzyme was followed by measuring the increase in the rate of hydrolysis of Ac-Tyr-OEt when acyl-chymotrypsin was incubated with this substrate[14].

The kinetic scheme for this reactivation process is the following:

$$ES' \xrightarrow{k_D} E + P'; \quad E + S \xrightarrow{k_{cat}} E + P \qquad \text{(Scheme 1)}$$

where ES' is any transient inactive acyl-enzyme, E is the free enzyme, S the substrate (*i.e.* Ac-Tyr-OEt) in excess; P' and P are the hydrolysis products. The free enzyme concentration E and the product concentration P are respectively equal to:

$$E = E_0 (1 - e^{-k_D t}) \qquad (1)$$

and

$$P = k_{cat} E_0 t + \frac{k_{cat}}{k_D} E_0 (e^{-k_D t} - 1) \qquad (2)$$

in which E_0 is the total enzyme concentration and t is time. For large values of t, the expression of P simplifies and becomes equal to:

$$P = k_{cat} E_0 t - k_{cat} E_0 / k_D \qquad (3)$$

In the plot of the appearance of P versus time, the asymptote to the linear section of the curve cuts the time axis for a value of t equal to $1/k_D$.

Inactivation of α-chymotrypsin by 2-bromomethyl-3,1-benzoxazin-4-one (Ib) or p-nitrophenyl N-(bromoacetyl) anthranilate (III). 100 μl of a freshly prepared solution of the Compd Ib or III (at the initial concentration 10 mM) in acetonitrile were added with swirling to a solution of α-chymotrypsin (5–10 μM) in 5 ml of 0.025 M HEPES–NaOH or MES–NaOH buffer (0.5 M NaCl; pH range 5.4–7.5) at 25 °C. After different incubation periods, the enzymatic activity of an aliquot (10 μl) of the mixture was

tested towards a specific substrate, Ac-Tyr-OEt (10 mM) in 5 ml of 0.1 M NaCl, pH 7.5, and 25 °C, and compared to that of a control.

The action of Compd Ib upon trypsin was studied in similar conditions but tryptic activity was assayed with Bz-Arg-OEt (10 mM) at pH 7.5 and 25 °C.

Reaction of Compd III with acetyl-chymotrypsin. α-Chymotrypsin (7 μM) was incubated at pH 5.3 (MES–NaOH buffer) and 25 °C in the presence of *p*-nitrophenyl acetate (0.2 mM) for 15 min. The acetyl-chymotrypsin formed had a half-life of about 2 h at this pH. Therefore reagent III (at the final concentration 0.195 mM) was added to the solution and after different incubation periods the activity of an aliquot was tested with Ac-Tyr-OEt (10 mM) at pH 7.5 and 25 °C. In these conditions the inactive acetyl-enzyme rapidly deacylated and the native enzyme was restored; the half-time of the acetyl-enzyme was about 2.5 min.

Preparation and properties of modified α-chymotrypsin. 25 mg of α-chymotrypsin were dissolved in 100 ml of MES–NaOH buffer (pH 5.6) at room temperature; therefore 1.25 ml of a solution of reagent Ib or III (at the initial concentration 20 mM) in acetonitrile were added to the enzyme solution and the mixture was gently stirred for about 4 h. After dialysis at 4 °C against 0.001 M HCl for 48 h, the solution was concentrated *in vacuo* with a Selectron ultrathimble purchased from Carl Schleicher and Schüll (Germany) and purified on a Sephadex G-25 column (32 cm × 1.2 cm) equilibrated with 0.001 M HCl. Fractions absorbing at 280 nm were gathered and concentrated again in vacuo with an ultrathimble against 0.001 M HCl. Finally the solution was passed through a 0.22-μm millipore filter and stored at 4 °C.

α-Chymotrypsin modified by radioactive Compd III (*p*-nitrophenyl *N*-(bromo-[2-^{14}C]acetyl) anthranilate was prepared as described above.

Radioactivity was determined by means of a Packard Tri-Carb liquid scintillation spectrometer (model 3375) in a water-miscible scintillation solution[18]. The extent of incorporation of the labeled reagent in the enzyme (exhaustively dialyzed against 0.001 M HCl) was estimated from a standard curve and by assuming, for the evaluation of the protein concentration, that the specific absorbance of the inhibited enzyme at 280 nm was the same as that of the untreated enzyme. For the determination of the standard curve the radioactive reagent was at first hydrolyzed in a slightly alkaline solution; the hydrolysis products were then soluble in the acidic aqueous solution added to the scintillation mixture.

The ultraviolet absorption spectra of the native and modified chymotrypsins were measured using a Cary model 14 recording spectrophotometer.

Their circular dichroism spectra were measured with a Roussel-Jouan dichrograph. Cell path lengths were 1 cm between 320 and 250 nm, and 0.05 cm between 260 and 215 nm. Protein concentrations were adjusted to 0.8–0.9 mg per ml.

The molecular weight of the inhibited enzyme was determined according to the procedure of Whitaker[19] using a column of Sephadex G-75 that had been calibrated with dextran blue 2000 (mol. wt $2 \cdot 10^6$), pepsin (mol. wt 35 000) and native α-chymotrypsin (mol. wt 25 000).

Amino acid analyses. Native or modified α-chymotrypsin was at first filtered through Sephadex G-25 to eliminate ninhydrin-positive low molecular weight impurities[20]. Therefore lyophilized samples of performic acid-oxidized protein (1–2 mg)[21] were mixed with constant-boiling hydrochloric acid, de-aerated and hydrolyzed in vacuo for 20 h at 110 °C. Hydrolysates were evaporated to dryness and the residue

taken up in 0.7 M sodium citrate buffer, pH 2.2. Amino acid analyses were carried out by the method of Spackman *et al.*[22], with a Beckman model 121 automatic amino acid analyzer coupled to an Infotronic model C.R.S. 100 A integrator. Molar ratios of amino acids were calculated on the basis of leucine = 19.0.

RESULTS

*Inactivation of α-chymotrypsin by 2-bromomethyl-3,1-benzoxazin-4-one (Ib) or p-nitrophenyl N-(bromoacetyl) anthranilate (III)**

α-Chymotrypsin was incubated in the presence of Reagent Ib (or III) in about 25-fold molar excess at pH 6 or 7 and 25 °C. The activity of a mixture aliquot was then tested at different incubation periods against Ac-Tyr-OEt at pH 7.5. The resulting kinetic run showed a lag period and the rate of hydrolysis of Ac-Tyr-OEt increased progressively to reach a maximal constant value after several minutes (Fig. 2). This phenomenon was likely due to the regeneration of the free enzyme from the acyl-enzyme formed during the reaction of Reagent Ib with α-chymotrypsin (see the experimental section for the kinetic analysis of the curves) and was less visible after long incubation periods.

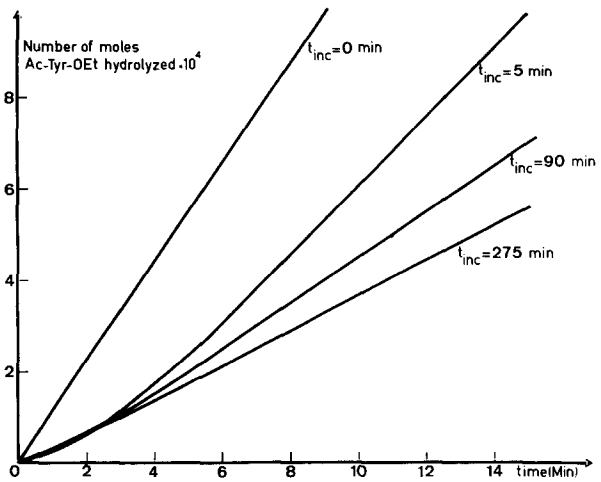


Fig. 2. Kinetic curves for the hydrolysis of Ac-Tyr-OEt by α-chymotrypsin incubated in the presence of 2-bromomethyl-3,1-benzoxazin-4-one (compd Ib) for different times (t_{inc}). Experimental conditions for the incubation: pH 7.05 (HEPES–NaOH buffer); temperature 25 °C; enzyme concentration, 7.6 μM; reagent concentration, 0.2 mM. Conditions for the kinetic test: pH 7.5; 25 °C; Ac-Tyr-OEt concentration, 10 mM; enzyme concentration, 0.015 μM.

* The spontaneous hydrolysis of the ester Compd III is very fast in aqueous solution between pH 5 and 7 and leads to the formation of the cyclic intermediate, Compd Ib (ref. 8). Compd III was then preincubated a few minutes before the addition of the enzyme so that the reaction medium contained only inactivator, Compd Ib. If this preincubation was not made, the rate of inactivation was only slower at low pH (pH 5.4 for instance) due to the limiting rate of hydrolysis of Compd III. There was no evidence for a direct reaction of Compd III with α-chymotrypsin as shown by the identical rates of *p*-nitrophenol release for the hydrolysis of Compd III (concentration 16 μM) in the absence and in the presence of α-chymotrypsin (concentration 70 μM) at pH 4.5 and 25 °C.

The maximal constant values of rates indicating that the deacylation of the acyl-enzyme approached completion were then compared, after different incubation times, to that one of a blank. The change in the activity of the enzyme as a function of time is given in Fig. 3. The activity drops rapidly in the first minutes of incubation, then decreases to a final value of 30–40% at incubation pH 6.0 or 7.0. A new addition of the Reagent Ib after 2.5 h of incubation does not reduce this activity.

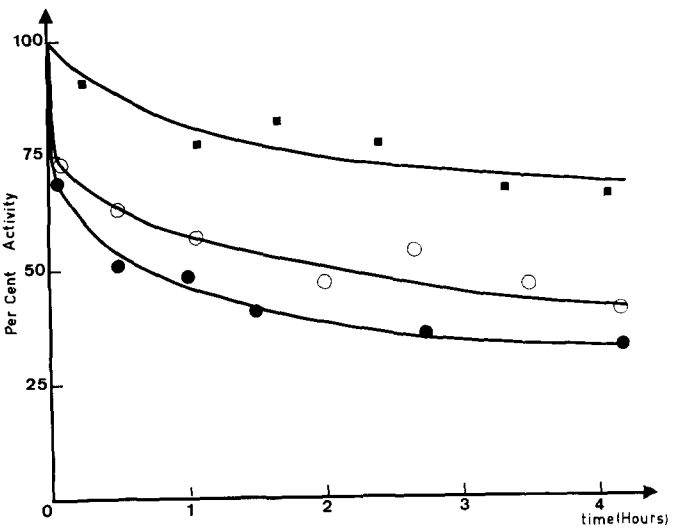


Fig. 3. Inactivation of α-chymotrypsin by Compds Ib or III as a function of time at pH 6.04 (○—○) or pH 7.02 (●—●) and 25 °C. The other experimental conditions are as described in Fig. 1. ■—■, inactivation of 5 μM α-chymotrypsin by 0.20 mM *N*-(bromoacetyl) anthranilic acid (Compd IIb) at pH 7.03 and 25 °C. It has been verified that the enzyme (7.5 μM) incubated for 4 h at pH 7 in the absence of reagent exhibits no significant loss of activity.

Under similar experimental conditions, the hydrolytic product of Reagent Ib, *N*-(bromoacetyl)anthranilic acid (IIb), inactivated α-chymotrypsin less rapidly (Fig. 3).

α-Chymotrypsin which had been acylated by *p*-nitrophenyl acetate at pH 5.3 (see the experimental procedure) was not inactivated by Reagent Ib at this same pH. The reactivation curves of the acetyl-chymotrypsin in the presence of Ac-Tyr-OEt at pH 7.5 were identical before and after addition of Reagent Ib (or III). A loss of activity of about 10% was only observed after 3.5 h incubation while, in the absence of *p*-nitrophenyl acetate, a loss of about 60% was found. Therefore, by protecting the active site of α-chymotrypsin, the inhibitory action of Reagent Ib is prevented.

Trypsin was not inactivated by 2-bromomethyl-3,1-benzoxazin-4-one (Reagent Ib) under similar experimental conditions, but an inactive transient enzyme-reagent complex was formed, as in the case of the reaction of Reagent Ib with α-chymotrypsin.

Comparative enzymatic hydrolyses of 2-methyl-3,1-benzoxazin-4-one (Ia) and 2-bromomethyl-3,1-benzoxazin-4-one (Ib)

De Jersey *et al.*[23,24] have shown that oxazolinones are good acylating agents for α-chymotrypsin. It might be then expected that 3,1-benzoxazin-4-ones Ia and Ib,

which have closed structures, could react with α-chymotrypsin due to their activated ester bond[25].

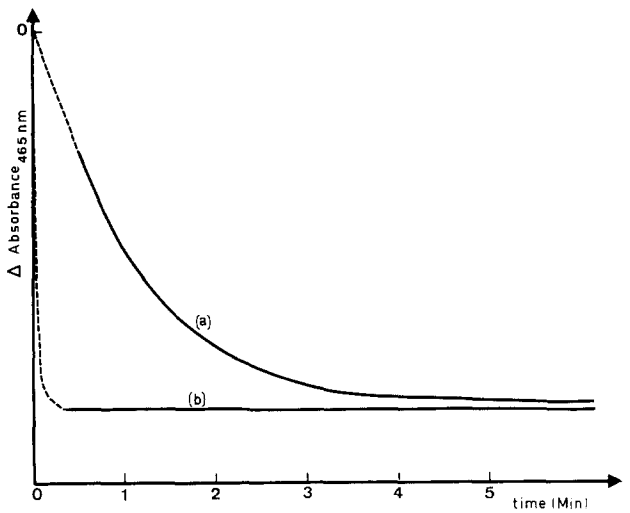


Fig. 4. Reaction of 2-methyl-3,1-benzoxazin-4-one (Compd Ia) (Curve a) and 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib) (Curve b) with α-chymotrypsin in the presence of proflavin. The experimental conditions were: enzyme concentration = 9.65 μM; proflavin concentration = 52 μM; substrate concentration = 95 μM; pH 7.08 (0.025 M HEPES–NaOH buffer; 0.5 M NaCl); 25 °C. The change in absorbance at 465 nm was about 0.1.

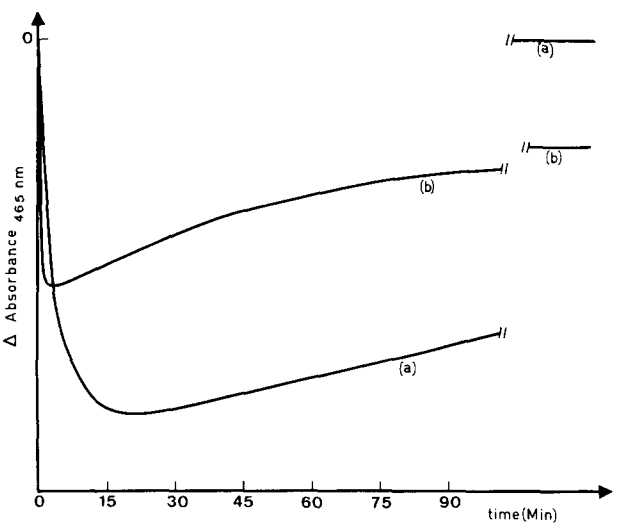


Fig. 5. Reaction of equivalents of 2-methyl-3,1-benzoxazin-4-one (Compd Ia) (Curve a) and 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib) (Curve b) with α-chymotrypsin in the presence of proflavin. The experimental conditions for the reaction of Compd Ia with the enzyme were: enzyme concentration = 18.7 μM; substrate concentration = 16.9 μM; proflavin concentration = 49.2 μM. For the reaction of Compd Ib with α-chymotrypsin, the reaction medium contained: enzyme concentration = 9.67 μM; substrate concentration = 9.33 μM; proflavin concentration = 51 μM. In both cases the medium pH was 7.52 (0.025 M HEPES–NaOH buffer; 0.5 M NaCl) at 25 °C. Plateau for Curve a was obtained after about 20 h and plateau for Curve b was obtained after about 200 min.

In Figs 4 and 5 are given the time-dependent changes in absorbance for the α-chymotrypsin-proflavin complex after addition of the 3,1-benzoxazin-4-ones Ia and Ib under different experimental conditions. As previously shown by Bernhard *et al.*[17], displacement of proflavin is related to the formation of an acyl-enzyme (Fig. 4), and regeneration of the original absorbance of the enzyme–dye complex is concomitant with the hydrolysis of the acyl-enzyme intermediate (Fig. 5). These processes may be described by the following scheme:

$$\begin{array}{ccccccc} & & & K_s & & k_2 & & k_3 & \\ & E & + & S & \rightleftharpoons & ES & \longrightarrow & ES' & \longrightarrow & E & + & P \\ +I & \Updownarrow & K_i & & & & & & & +I & \Updownarrow & K_i \\ & EI & & & & & & & & & EI & \end{array} \qquad \text{(Scheme 2)}$$

in which E is the enzyme, S the substrate, I proflavin and P the reaction product; ES is the Michaelis complex, ES' is the acyl-enzyme and EI the enzyme–proflavin complex.

The specific rate constants for acylation (k_2/K_s) of α-chymotrypsin by 3,1-benzoxazin-4-ones and for deacylation (k_3) of the corresponding acyl-chymotrypsins have been determined in the presence of proflavin and are given in Table I.

TABLE I

KINETIC CONSTANTS FOR THE HYDROLYSIS OF 2-METHYL-3,1-BENZOXAZIN-4-ONE (Ia) AND 2-BROMOMETHYL-3,1-BENZOXAZIN-4-ONE (Ib) BY α-CHYMOTRYPSIN

k_2/K_s was determined by the proflavin displacement method[17] in the conditions given in Fig. 4 at pH 7.08 and 25 °C. k^*_D was determined by displacement of proflavin[17] in the conditions given in Fig. 5 at pH 7.52 and 25 °C. k_3 was determined in the presence of a specific substrate, Ac-Tyr-OEt (10 mM), at pH 7.50 and 25 °C.

Substrate	k_2/K_s ($M^{-1}\cdot s^{-1}$)	k_3 (s^{-1})	k^*_D (s^{-1})
Ia	160	$5\cdot 10^{-5}$	$3\cdot 10^{-4}$
Ib	>160	$3\cdot 10^{-4}$*	$5\cdot 10^{-3}$*

* Mean values for deacylations of the unmodified and modified acyl-enzymes (complexes ES' and ES'' in Scheme 3).

In the reaction of equivalents of 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib) and α-chymotrypsin, the final absorbance of the enzyme-proflavin complex after deacylation of the acyl-enzyme (Fig. 5; Curve b) is not identical to the initial one before the addition of the reagent to the medium. This result shows that the enzyme has been modified and its ability to bind proflavin has decreased. Therefore Scheme 2 must be modified in this case and must include another enzyme–reagent complex ES'' which is likely formed from the acyl-enzyme ES' and the deacylation of which leads to the modified enzyme E':

$$\begin{array}{ccccccccccc} & EI & & & & & & & EI & & \\ +I & \Updownarrow & K_i & & & & & +I & \Updownarrow & K_i & \\ & E & + & S & \rightleftharpoons & ES & \longrightarrow & ES' & \longrightarrow & E & + & P \\ & & & & & & & \downarrow & k_3' & & & \\ & & & & & & & ES'' & \longrightarrow & E' & + & P \\ & & & & & & & & +I & \Updownarrow & K_i' & \\ & & & & & & & & & E'I & & \end{array} \qquad \text{(Scheme 3)}$$

The deacylation rate constants of these acyl-chymotrypsins in the presence of Ac-Tyr-OEt have also been measured (k_D values in Table I) and have been found several times higher. The same phenomenon was noted by Bender *et al.*[26] for the deacylation of acetyl-chymotrypsin in the presence and in the absence of Ac-Tyr-Oet.

Stoichiometry of the inactivation reaction

Incubation experiments with *p*-nitrophenyl *N*-(bromo[2-^{14}C]acetyl) anthranilate in 25-fold molar excess show that 1.20 $\pm$ 0.1 molecules of the labeled reagent have been incorporated per molecule of protein. This result indicates that the reaction is nearly stoichiometric.

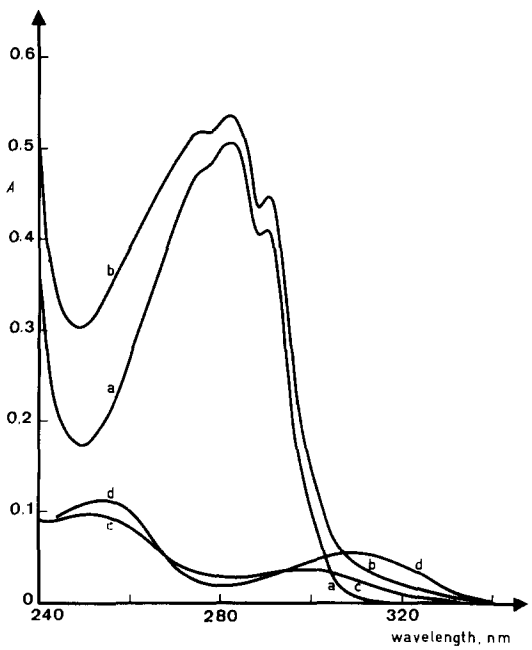


Fig. 6. Ultraviolet absorbance spectra of α-chymotrypsin (10 μM) in 0.001 M HCl (Curve a), α-chymotrypsin modified by 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib) in 0.001 M HCl (Curve b), *N*-(bromoacetyl)anthranilic acid (10 μM) in 0.001 M HCl (Curve c) and in pure dioxane (Curve d).

Molecular properties of the modified α-chymotrypsin

The ultraviolet absorption spectrum of the modified enzyme (in 0.001 M HCl) is significantly different from that of the native enzyme (Fig. 6). In the wavelength range 310–340 nm a weak absorbance is recorded; furthermore the ratio of absorbance at 280:250 nm decreases from 2.85 in the native enzyme to about 1.8 in the inhibited enzyme. The spectrum of the modified enzyme is approximated by a linear combination of those of the native enzyme and *N*-(bromoacetyl) anthranilic acid (Compd IIb)*.

* At 280 nm, the absorbance of the reagent itself in the modified enzyme is estimated to be about 5% of the total absorbance of the protein. Therefore, as a first approximation, the extinction coefficients of native and modified enzymes at 280 nm are assumed to be similar for calculations of protein concentrations.

The circular dichroism spectra of native and modified α-chymotrypsins in 0.001 M HCl and at neutral pH are compared between 320 and 220 nm in Figs 7 and 8. Both spectra are characterized by the same negative maxima centered at 305, 262 and 230 nm. Moreover two positive maxima separated by a negative minimum occur at 298 and 288 nm. These spectra are comparable to those previously reported[27,28]. However, the circular dichroism spectrum of the modified α-chymotrypsin is more intense than that of the native enzyme between 280 and 250 nm. The intensities of

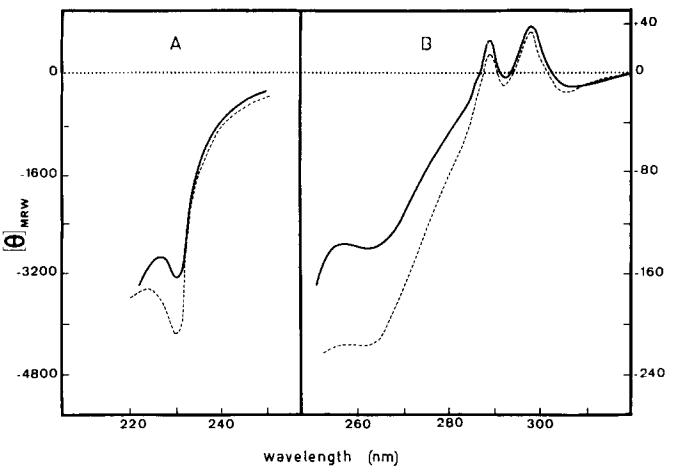


Fig. 7. Circular dichroism spectra of native α-chymotrypsin (———) and modified α-chymotrypsin (— — —) in 0.001 M HCl. Enzyme concentrations are estimated from absorbance measurements at 280 nm by assuming the same extinction coefficient for both proteins: they are 0.95 mg/ml. Cell path lengths are: A, 0.05 cm; B, 1 cm. The molar ellipticities are on a mean residue basis.

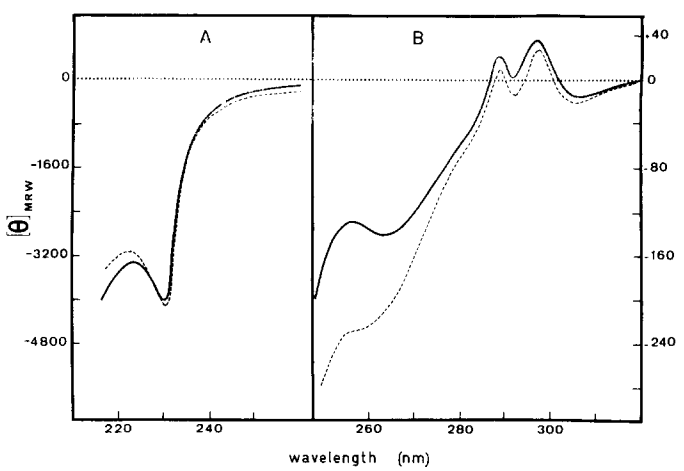


Fig. 8. Circular dichroism spectra of native α-chymotrypsin (———) and modified α-chymotrypsin (— — —) at pH 7 in 0.066 M phosphate buffer. Enzyme concentrations are estimated from absorbance measurements at 280 nm by assuming a same extinction coefficient for both proteins; they are 0.85 mg/ml. Cell path lengths are: A, 0.05 cm; B, 1 cm. The molar ellipticities are on a mean residue basis.

the negative dichroic band of modified and native enzymes at 229–230 nm are also markedly different in 0.001 M HCl (Fig. 7) but not at neutral pH (Fig. 8).

The molecular weight of the inhibited enzyme determined on a column of Sephadex G-75 is indistinguishable from that of the native enzyme and there is no evidence of dimeric and oligomeric species (Fig. 9).

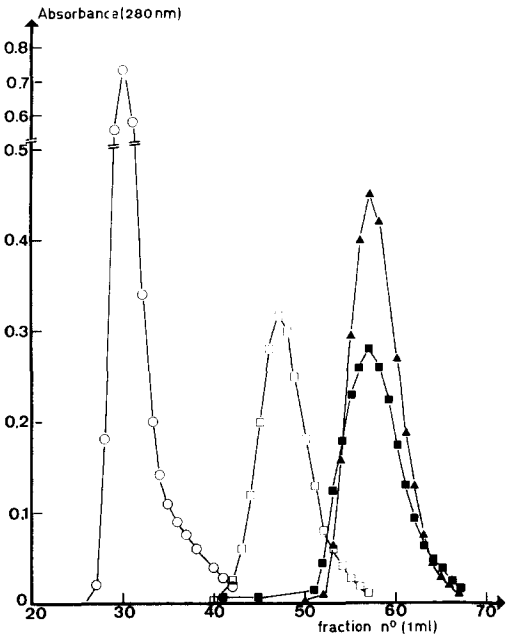


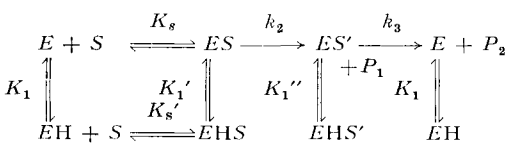
Fig. 9. Gel filtration of dextran blue (○—○), pepsin (2 mg/ml) (□—□), modified α-chymotrypsin (■—■), native α-chymotrypsin (2 mg/ml) (▲—▲), on a column (1.4 cm × 52 cm) of Sephadex G-75 equilibrated and eluted with 0.05 M sodium acetate buffer (pH 5.6). The rate of elution is 11 ml/h at 22–24 °C.

Catalytic activity of the modified enzyme

The active site in α-chymotrypsin inactivated by Compd Ib is almost intact, as is shown by titration of the modified enzyme with *p*-nitrophenyl acetate[16]. The percent of active sites is found to be equal to about 75 instead of 90 for the native enzyme.

The kinetic constants K_m and k_{cat} for the native and modified α-chymotrypsin-catalyzed hydrolyses of different substrates at pH 8 (or 7.5) are given in Table II. The Michaelis constant K_m of the modified enzyme increases several times over that of the native enzyme and its k_{cat} is similar or slightly smaller.

The pH dependence of the kinetic constants k_{cat} and K_m for the modified α-chymotrypsin-catalyzed hydrolysis of Ac-Tyr-OEt between pH 6 and 8 is shown in Fig. 10. These kinetic data are analyzed from the following reaction scheme[29]:



(Scheme 4)

TABLE II

Kinetic constants for the hydrolysis of various substrates by native α-chymotrypsin and α-chymotrypsin modified by 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib), at 25 °C.

Substrate	*Enzyme*	k_{cat}* (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
p-Nitrophenyl acetate**	native	$5.6 \cdot 10^{-3}$	$3 \cdot 10^{-6}$	$1.9 \cdot 10^{3}$
	modified	$5.6 \cdot 10^{-3}$	$1.35 \cdot 10^{-5}$	$4.2 \cdot 10^{2}$
Ac-Tyr-OEt***	native	190	$9.6 \cdot 10^{-4}$	$2 \cdot 10^{5}$
	modified	97	$3.7 \cdot 10^{-3}$	$2.6 \cdot 10^{4}$
Ac-Trp-OEt	native†	44.9	$1.2 \cdot 10^{-4}$	$3.65 \cdot 10^{5}$
	modified††	37.5	$2.1 \cdot 10^{-4}$	$1.8 \cdot 10^{5}$

* After correction for 100 percent of active sites.

** 0.025 M HEPES–NaOH buffer; 0.5 M NaCl; pH 7.55; 4.6% (v/v) acetonitrile–water; substrate concentration, 3–270 μM.

*** No buffer (potentiometric measurements); 0.1 M NaCl; pH 8; substrate concentration, 0.5–20 mM.

† Ref. 29; pH 8.18; 0.1 M Tris–HCl; 0.81% (v/v) acetonitrile–water.

†† No buffer (potentiometric measurements); 0.1 M NaCl; pH 8; substrate concentration, 0.1–2 mM.

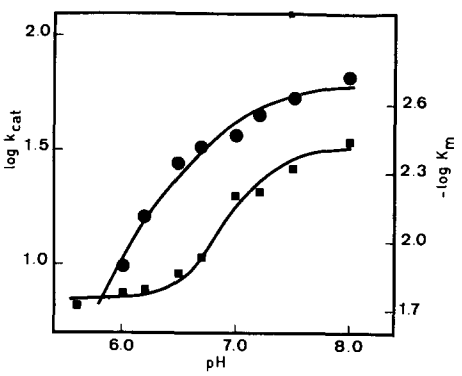


Fig. 10. Kinetic parameters for the modified α-chymotrypsin-catalyzed hydrolysis of Ac-Tyr-OEt as a function of pH at 25 °C; ●—●, log k_{cat} (s^{-1}); ■—■, $-\log K_m$ (M).

where S is the substrate; E and EH are the active and inactive forms of the enzyme; ES and EHS, the corresponding enzyme-substrate complexes; ES' and EHS', the different forms of the acyl-enzyme intermediate; P_1 and P_2, the hydrolysis products. Under steady state and conditions where the initial concentration of the substrate S_0 is very much larger than that of the enzyme E_0, the initial reaction rate is given by the expression:

$$v = \frac{k_{cat(pH)}\, S_0\, E_0}{K_{m(pH)} + S_0} \tag{4}$$

with

$$k_{cat(pH)} = \frac{k_2 k_3}{k_2\,(1 + H^+/K_1'') + k_3\,(1 + H^+/K_1')} \tag{5}$$

and

$$K_{m(pH)} = \frac{k_3\,(1 + H^+/K_1)\, K_s}{k_2\,(1 + H^+/K_1'') + k_3\,(1 + H^+/K_1')} \tag{6}$$

The rate-determining step for the hydrolysis of specific ester substrates by native α-chymotrypsin is deacylation[30]. As the kinetic constant k_{cat} for the modified enzyme at optimal pH is practically identical to that for the native enzyme (Table II), the deacylation step is also limiting in the hydrolysis of specific substrates by the modified chymotrypsin. The expressions of $k_{cat(pH)}$ and $K_{m(pH)}$ simplify and become, respectively, equal to:

$$k_{cat(pH)} = \frac{k_3}{1 + H^+/K_1''} \tag{7}$$

and

$$K_{m(pH)} = \frac{1 + H^+/K_1}{1 + H^+/K_1''} \cdot \frac{K_s k_3}{k_2} \tag{8}$$

From the experimental data on the variation of $k_{cat(pH)}$ with pH (Fig. 10), a pK_1'' of 6.7 at 25 °C is determined. This value is identical to that measured in the native α-chymotrypsin-catalyzed hydrolysis of Ac-Tyr-OEt[31].

The kinetic constant $K_{m(pH)}$ is found to be pH dependent between pH 6 and 8 (Fig. 10). Dixon's method[32] is used to analyze the plot of pK_m versus pH and values of pK_1 and pK_1'' are found to be equal to 7.2 and 6.6, respectively. In contrast, the Michaelis constant K_m of the native chymotrypsin is constant in this pH range and pK_1 is equal to 6.7 (ref. 31).

This increase in the pK_1 value for the modified enzyme may result from the interaction of the negative charge (carboxylic group) of the reagent bonded to the enzyme with the positive charge of the active histidine-57. An analogous interaction has been postulated by Hille and Koshland[33] in α-chymotrypsin labeled with 2-acetamido-4-nitrophenol.

TABLE III

Inhibition constants of competitive inhibitors towards native α-chymotrypsin and α-chymotrypsin modified by 2-bromomethyl-3,1-benzoxazin-4-one (compd Ib), at pH 8 and 25 °C.

Inhibitor	*I* ($M \times 10^3$)	K_i ($M \times 10^3$)* *(modified enzyme)*	K_i ($M \times 10^3$) *(native enzyme)*
Benzamide	21.3	16.2	8.86**
Indole	2	2.33	0.97**
Proflavin	0.056	0.155	0.037***

* The concentration of Ac-Tyr-OEt was varied between 0.5 mM and 10 mM.

** Ref. 34. Experimental conditions: pH 7.91 (0.1 M Tris–HCl); 25 °C; 3.4% (v/v) acetonitrile–water.

*** Ref. 17. Experimental conditions: pH 8.0; 25 °C; 0.1 M KCl.

Finally the inhibition constants for different competitive inhibitors were determined by using Ac-Tyr-OEt as substrate at pH 8 and the results obtained are given in Table III. The values of the inhibition constants are larger in the case of the modified enzyme than for the native enzyme and the observed variations seem to be related to the size of the inhibitors.

Amino acid analyses of the inactivated enzyme

Amino acid analyses of the native α-chymotrypsin after performic acid oxidation and acidic hydrolysis indicate that both methionine residues of the molecule are quantitatively converted to methionine sulfones. Similar treatment of the modified chymotrypsin yields only one methionine sulfone/mole enzyme. In this last case, nor methionines neither eventual decomposition products of the alkylated methionine[35] can be detected in significant amounts. No other change in amino acid composition is observed.

Since alkylated methionines are stable to performic acid oxidation, the above result indicates that one of both methionine residues in chymotrypsin has been modified by 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib).

Location of the modified residue

Sigman *et al.*[36,37] have shown that α-bromo-4-nitroacetophenone alkylates the methionine-192 of α-chymotrypsin. The alkylated enzyme exhibits an intense optically active absorption band in the region 290–365 nm which is not present in the spectra of the native enzyme or in those of the reagent, and which is likely due to the formation of a sulfonium ylide on the enzyme.

The modified enzyme (10–20 μM) is then incubated at pH 6 (0.025 M MES–NaOH buffer; 0.5 M NaCl) in the presence of α-bromo-4-nitroacetophenone (0.2 mM) and the ultraviolet absorption spectrum of the mixture is recorded at different incubation periods (0–1 h). For the incubation of this alkylating reagent with the modified enzyme there is no appearance of a new absorbance band in the region 290–365 nm, contrary to what is observed with the native chymotrypsin. Thus α-chymotrypsin previously inactivated by Compd Ib does not react with α-bromo-4-nitroacetophenone and this result suggests that both these reagents, 2-bromomethyl-3,1-benzoxazin-4-one and α-bromo-4-nitroacetophenone, alkylate the same residue of the enzyme (methionine-192).

DISCUSSION

2-Bromomethyl-3,1-benzoxazin-4-one (Compd Ib) irreversibly and partly inactivates α-chymotrypsin but not trypsin at neutral pH. This bifunctional reagent carries in its structure an acylating function and an alkylating one. In the first step (see Fig. 11) it acylates the active serine of the enzyme (serine 195) as shown by the method of proflavin displacement. This behavior is expected in regard to the fact that oxazolinones which have a closed structure are good enzyme acylating agents*. Then, after formation of this rather stable acyl-enzyme intermediate, intramolecular alkylation of an enzymic residue occurs. Finally the alkylated acyl-enzyme

* The values of the second-order acylation rate constants k_2/K_s (equal to k_{cat}/K_m) for the α-chymotrypsin-catalyzed hydrolyses of 2-phenyl-oxazolin-5-one[24] and 2-methyl-3,1-benzoxazin-4-one (Compd Ia) are equal to $6.6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ (at 25 °C and pH 6.96) and 160 $M^{-1} \cdot s^{-1}$ (at 25 °C and pH 7.08; measured by the indirect proflavin displacement method), respectively. The observed difference in these k_2/K_s values is mainly due to the high reactivity of the oxazolinone carbonyl carbon towards nucleophilic attack. Thus the rate constants for the alkaline hydrolyses of 2-phenyl-oxazolin-5-one[38] and Compd Ia[25] are equal to about $3 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (calculated from the value of k_{obs} at pH 9.52 and 30 °C and corrected for the ionization of the oxazolinone in this pH range ($pK_a = 9.4$)) and 56.2 $M^{-1} \cdot s^{-1}$ (at 25 °C), respectively.

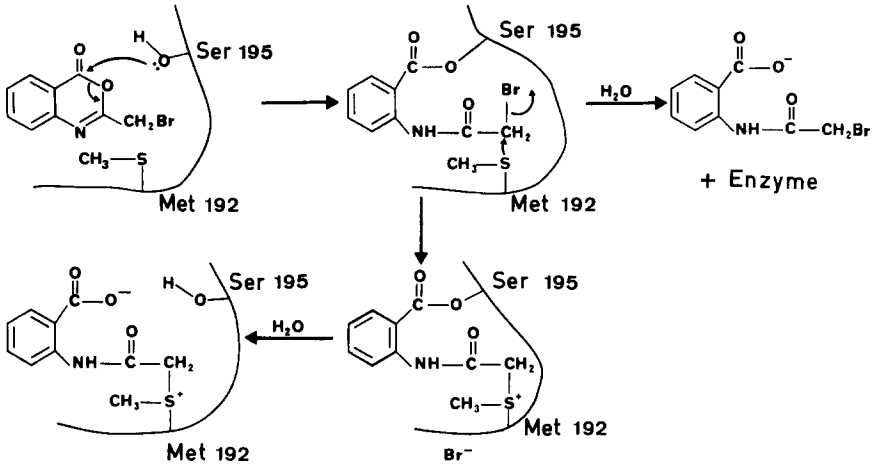


Fig. 11. Scheme of inactivation of α-chymotrypsin by 2-bromomethyl-3,1-benzoxazin-4-one (Compd Ib).

deacylates to give a stoichiometrically labeled enzyme whose catalytic properties are modified. If the active site is protected by acetylation of the active serine, the action of the reagent is prevented.

Amino acid analyses of the modified enzyme after performic acid oxidation and acid hydrolysis reveals loss of one methionine residue. This residue is likely to be methionine-192 which is known to be preferentially labeled by alkylating reagents[39]. Moreover, the modified enzyme does not react with α-bromo-4-nitroacetophenone which has been shown to react selectively with methionine-192.

The mechanism of action of Compd Ib is comparable to that of *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (Compd IV), but the modified enzymes have different catalytic properties. Thus alkylation of α-chymotrypsin by the Compd Ib does not markedly change the kinetic constant k_{cat} but increases the Michaelis constant K_m two-to five-fold, while alkylation by Compd IV increases k_{cat} from three- to eight-fold and also appreciably changes K_m (ref. 34). The catalytic activity of the enzyme modified by Compd Ib is rather analogous to that of the enzyme inactivated by alkylating reagents having an aromatic residue in their structure. In this last case K_m is mainly modified and increases several times[39] while k_{cat} remains pratically constant or decreases[39,40].

From crystallographic data, Steitz *et al.*[41] have indicated that methionine-192 appears to function as a flexible hydrophobic lid on the specificity pocket which is involved in substrate binding. When this methionine is alkylated by a bulky aromatic alkylating agent, the binding of a specific substrate or a competitive inhibitor on the enzyme is then sterically hindered, as shown by increased K_m and K_i values. Thus Gerig *et al.*[42,43] have suggested from nuclear magnetic resonance measurements that α-chymotrypsin alkylated by trifluoromethyl-substituted α-bromoacetanilides does not bind L-tryptophan because of a mutual exclusion effect.

Circular dichroism spectra of the modified α-chymotrypsin at acidic or neutral pH show a more intense negative dichroic band centered at 260 nm than that of the native enzyme. As the α-chymotrypsin modified on methionine-192 contains a

reagent-derived chromophore which absorbs in the investigated wavelength range (and carries an asymmetrical sulfur atom), direct reciprocal perturbations of some residues of the enzyme protein and the derivative moiety may be responsible for this change in the circular dichroism spectra. Furthermore, depending on the medium pH, the negative dichroic band of the modified enzyme at 230 nm is more intense than that of the native protein; this difference is observed at acidic pH but not at neutral pH. It is known that the conformation of α-chymotrypsin changes in the acidic pH range due to the disruption of the salt bridge isoleucine-16–aspartic acid-194; consequently, the optical rotatory dispersion curves of the native enzyme or the circular dichroism spectra change with pH between pH 2 and 7 (ref. 44). Therefore it may be assumed that the increase in the negative molar ellipticity at 230 nm displayed by the modified enzyme at acidic pH (Fig. 7) arises from a stabilization of the protein conformation in the neutral form. Ghelis *et al.*[45,46] have previously indicated that alkylation or oxidation of the methionine-192 causes a displacement of the pK of the isoleucine-16 dependent structural transition in the alkaline pH range and stabilizes the enzyme in the neutral form.

Thus circular dichroism measurements show that the 2-acetamidobenzoic acid moiety of the modifier interacts at least partly with the enzyme surface and probably with the specificity pocket.

ACKNOWLEDGMENTS

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