METHYL-1-METHYL-3,4-DIHYDRO-β-CARBOLINE-3-CARBOXYLATE AS A NEW CHROMOGENIC SUBSTRATE FOR α-CHYMOTRYPSIN.*

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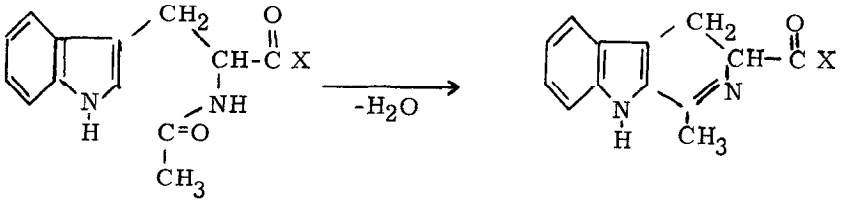
SUMMARY - α-Chymotrypsin catalyses the hydrolysis of methyl-1-methyl-3,4-dihydro-β-carboline-3-carboxylate while the corresponding amide is completely unreactive. The reaction can be followed conveniently by colorimetric procedure and the enzyme exhibits a marked preferentiality for the D-isomer of the ester.

It has been recently suggested (1, 2) that an intramolecular reaction of the substrate can constitute an important step in the mechanism of action of α-chymotrypsin. A consequence of this "substrate activity" in the specially organized enzyme-substrate complex is that some modulation in enzymatic action could be realized by specific modification of the substrate molecule.

This approach potentially leads, on the one hand, to useful information on the enzymatic mechanism and, on the other, to the discovery of new classes of substrates.

In this paper we wish to report on the α-chymotrypsin catalyzed hydrolysis of the cyclodehydrated form (3,4-dihydro-β-carboline-3-carboxylic acid derivatives**) of N^{α} acetyl tryptophan ester, which can also be proposed as



a new chromogenic substrate.

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** Abbreviations used :
MDC = 1-methyl-3,4-dihydro-β-carboline-3-carboxylic acid.
MDCOMe = methyl-1-methyl-3,4-dihydro-β-carboline-3-carboxylate.
TFA = trifluoroacetic acid.

EXPERIMENTAL -

Bovine α-chymotrypsin (3xcryst.) was purchased from the Worthington Biochem. Corp. and all other chemicals were of the best grade available. Ultraviolet spectra were recorded with a Cary model 15 spectrophotometer.

Preparation of the substrate

A solution of D-tryptophan (2 g) in TFA (25 ml) was treated with acetyl chloride (1.5 ml) at room temperature. After 15-20 min water (1 ml) was added and the solution evaporated to dryness under reduced pressure. The oily residue was dissolved in boiling water (20 ml) and MDC hydrochloride separates as a yellow crystalline product by standing at room temperature. The hydrochloride was dissolved in about 50 ml of 0.2 N HCl and the pH value was increased to 4.5 by addition of sodium acetate. The free MDC, which rapidly separates, was collected by filtration and dryed over KOH pellets. Yield 1.15 g.

MDC (0.5 g) was allowed to stand overnight in about 10 ml of methanol : thionyl chloride (9:1, v/v) at room temperature. The solution was evaporated to dryness under reduced pressure and MDCOMe hydrochloride was crystallized from methanol/ethylether. Yield 0.48 g ; m.p. 175° ; $[\alpha]_D^{20}$ -227 (c = 0.5 N HCl.)

Anal. Calcd. for $C_{14}H_{14}N_2O_2.HCl$: C, 60.32 ; H, 5.06 ; N, 10.05
Found : C, 61.44 ; H, 5.22 ; N, 9.96

The amide was obtained by treating the ester with a saturated solution of ammonia in methanol for 48 h. at room temperature. Needles from methanol /ethyl ether ; m.p. 185°.

Anal. Calcd. for $C_{13}H_{13}N_2O.HCl$: C, 59.20 ; H, 4.96 ; N, 15.93
Found : C, 60.10 ; H, 5.13 ; N, 15.65

Measurement of the enzyme activity

The hydrolysis of the substrates was measured by following the increase of absorbancy at 352 nm for the transformation of the ester or the amide to the acid (Fig. 1). The differential extinction coefficient being pH dependent with a maximum at about pH 8 (Table) it is possible to follow the conversion by continuous measurements with the differential spectral technique (3). When less favorable pHs values were to be examined, suitable samples of the digestion mixture were diluted at different times with urea 6M (in Tris-HCl buffer 2M, pH 8) to stop the enzymatic reaction and the ΔO.D. at 352 nm re-

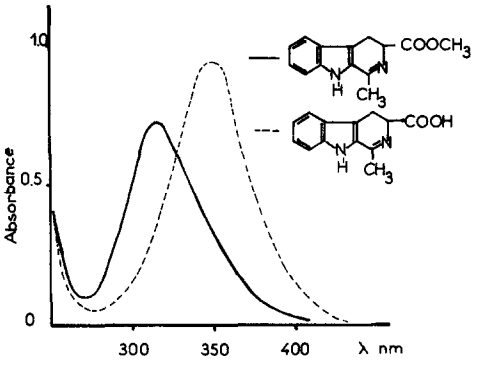

Fig. 1 Absorption spectra of MDC ($5x10^{-5}$M) and of MDCOMe ($5x10^{-5}$M) in aqueous solution at pH 8.

TABLE

pH	6.5	7.0	7.3	7.5	7.7	8.0	8.5	9.0
log $\Delta \varepsilon_{352nm}$	3.00	3.73	3.88	3.95	3.99	4.07	4.08	3.90

Difference molar absorbance ($\varepsilon_{MDC} - \varepsilon_{MDCOMe}$) at different pH values.

corded. The observance of Beer's law was checked, and the initial and infinite absorbances were used to check the stoichiometry of the reaction. Chromatographic controls showed that only the acid derivative was present at the end-point of the reaction.

Solutions of enzyme and substrates were in all cases freshly prepared : the ester substrate concentration in assay mixture was 0.05 to 1 x 10^{-3}M, the enzyme concentration 10^{-5} to 10^{-7}M, the higher concentration being used at the extreme pHs where the rate constants are smaller. The amide substrate concentration in the assay mixture was 10^{-3} to 10^{-6}M, the enzyme concentration 10^{-4} to 10^{-7}M. The kinetic constants were calculated from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

The synthesis of optically active MDC proceeds through a 2-acylation of

tryptophan indole ring followed by an intramolecular condensation reaction between the 2-ketonic function with the α-amino group. A detailed study on this new tryptophan modification will be published elsewhere (4).

The MDCOMe structurally differs from a specific substrate for α-chymotrypsin, such as methyl N^{α} acetyl tryptophanate because the aromatic moiety of the molecule is rigidly fixed to the sensitive ester function and to the α amino group position. Furthermore the α acyl amino group has completely disappeared in the MDCOMe which can be regarded as the product of the intramolecular dehydration of methyl N^{α} acetyl tryptophanate. The most interesting effects of these differences on the chymotryptic hydrolysis are :

I) While α-chymotrypsin catalyzes the hydrolysis of both the ester and the amide derivative of N^{α} acetyl tryptophan, the same enzyme exhibits only an esterasic activity when tested with β carboline derivatives. MDCOMe is hydrolyzed at an optimum pH values of 6.5 which is more acidic if compared with the one of methyl N^{α} acetyl tryptophanate (5). The K_m (app.) of the two substrates are of the same order of magnitude (Fig. 2) while the k_{cat} for MDCOMe (2.6 sec^{-1}) is about ten times lower. Other properties of MDCOMe as a substrate for α-chymotrypsin are summarized in the diagram of Fig. 2.

Despite this marked ability of MDCOMe to behave as a substrate for α-chymotrypsin the corresponding amide derivative is not hydrolyzed at any enzyme or substrate concentrations tested in a pH range from 5 to 9.

The possibility to modulate the enzymatic activity by modification of the substrate molecule is additional evidence to the effect that, at least in the case of α-chymotrypsin, the mechanism of the bond breaking process is more dependent on the structure of the substrate than generally admitted. In other words some functional groups responsible for the enzymatic catalysis are to be found in the substrate molecule itself when the specially organized enzyme -substrate complex is considered (1,2).

II) The high degree of L-specificity in the chymotryptic hydrolysis of methyl N^{α} acetyl tryptophanate disappears while a marked D- preferentiality appears in the case of MDCOMe. Indeed, the L- isomer of MDCOMe can be utilized as a substrate but its reactivity is smaller than the one of the D- isomer. This result has already been observed with other sterically restricted substrates (6,7) which were examined in order to study the geometry of the α-

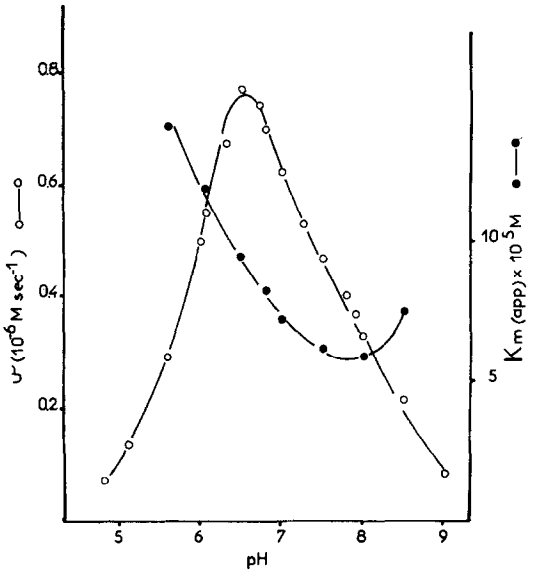

Fig. 2 The α-chymotrypsin catalyzed hydrolysis of MDCOMe at 25° in aqueous solution. $[E] = 0.3 \times 10^{-6}$M.

chymotrypsin binding site.

The peculiar behaviour of 3,4-dihydro-β-carboline-3-carboxylic acid derivatives as substrates together with their favorable chromogenic properties allow one to presume that they will be molecules of choice for studies involving α-chymotrypsin.

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