STEREOSPECIFICITY OF α-CHYMOTRYPSIN. CHANGES OF SUBSTRATE CONFIGURATION DURING ENZYMATIC HYDROLYSIS *

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1. Introduction

The stereospecificity of α-chymotrypsin is commonly considered as a kinetic phenomenon since the carboxyl derivatives of L-aromatic amino acids are hydrolyzed much more rapidly than their D enantiomers, which usually act as inhibitors. Although inversion of antipodal specificity has been observed with some sterically restricted molecules [1–3] no inversion of the substrate configuration has ever been denounced during the course of enzymatic reaction.

We wish to report in this paper on a racemase activity of α-chymotrypsin observed with cyclic substrates and to discuss the enzymatic and non-enzymatic mechanisms by which an inversion of steric configuration of the substrate can occur during the enzymatic hydrolysis.

2. Experimental

2.1. *Materials*

2.1.2. 2-Phenyl-4-benzyl-thiazolin-5-one

Thiobenzoyl DL-phenylalaninamide [4] (1 g) was dissolved in formic acid and after a few minutes at room temperature the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate, washed with water and the 2-phenyl-4-benzyl-thiazolin-5-one crystallized from ethyl acetate/petroleum ether. Yield: 0.75 g; m. pt. : 134–135°C. Analysis – Calculated for $C_{16}H_{13}NOS$: C, 71.88; H, 4.90; N, 5.24; S, 11.99. Found : C, 71.67; H, 5.04; N, 5.42; S, 11.50. The other thiazolinones listed in table 1 were obtained from the corresponding thiobenzoyl amino acid amides [4] by anhydrous acid treatment [5,6] and utilized without further purification.

2.1.2 L- and D-1-methyl-3,4-dihydro-β-carboline-3-carboxylic acids (MDC)*

The acids were synthesized from L- and D-tryptophan and acetyl chloride in trifluoroacetic acid [7]. The methyl esters (MDCOMe)* [3] were fixed by an overnight treatment of the acids with methanol:thionyl chloride 9:1 and crystallized as hydrochlorides from methanol ethyl ether.

Bovine α chymotrypsin (3 X cryst.) was purchased from Worthington Biochem. Corp. and all chemicals were the best available commercially.

2.2. *Kinetic measurements*

2.2.1. Hydrolysis

Kinetics of hydrolysis were followed spectrophotometrically using a Beckman DB and a Cary model 15 equipped with a thermostated cell compartment. The hydrolysis of thiazolinones was followed at 365 nm (fig. 1), and of MDCOMe at 352 nm [3]. The

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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.

Table 1
The kinetics of the α-chymotrypsin catalyzed hydrolysis of phenylthiazolinone derivatives of: (a) phenylalanine at different pH values; (b) other amino acids at pH 6.6.

(a) $k_{cat}/K_M \times 10^{-2}$ $M^{-1}.sec^{-1}$	2.50	3.30	2.84	1.57	0.85	0.32
pH	6.2	6.6	7.0	7.4	7.8	8.4
(b) $k_{cat}/K_M \times 10^{-2}$ $M^{-1}.sec^{-1}$	9.60	64.00	0.035	0.11	0.07	
Thiazolinones derivatives of:	Tyr	Trp	Ala	Val	Arg	

spontaneous hydrolysis is relatively slow and was taken into account only when greater than 3–5% of the total reaction.

The substrate concentration in the assay mixture was 0.5 to 1×10^{-4} M; the enzyme concentration 10^{-5} to 10^{-8} M; the kinetic constants were calculated from Lineweaver–Burk plots [8].

2.2.2. Optical rotation

Kinetics of optical rotation changes were followed at 436 nm using a Roussel–Jouan polarimeter digital, type 71, equipped with a thermostated cell compartment.

In a typical experiment 30 μmoles of substrate dissolved in 0.5 ml methanol were diluted with 9.5 ml of suitable buffer containing or not containing the enzyme. Optical rotation changes were followed as a function of time and compared with blanks of enzyme alone when necessary. The spontaneous racemization constant (K', see Results and discussion) is a first order constant.

3. Results and discussion

The 2-phenyl thiazolinones derived from aromatic amino acids are hydrolyzed by α-chymotrypsin to N^{α}thiobenzoyl amino acids and the reaction can be spectrophotometrically followed at 365 nm without interference from the enzyme and the reaction product (fig. 1). The rate of hydrolysis plotted against the pH depicts a bell-shaped curve with a maximum around 6.5 (table 1). The K_{cat}/K_M values reported in table 1 show that the phenyl thiazolinone derived from aromatic amino acids can be considered as specific substrates for α-chymotrypsin and the relative specificity observed with thiazolinone derivatives of different amino acids is the same currently observed with the classical synthetic substrates.

Although thiazolinones are utilized as a racemic mixture, they are completely hydrolyzed by α-chymotrypsin and no rate changes are observed during the reaction (fig. 2). Polarimetric measurements show that optical activity appears during the enzymatic hydrolysis and this activity is totally imputable to the optically active thiobenzoyl L amino acid which constitutes the single product at the end point of the reaction (fig. 2) as confirmed by comparison with a synthetic product.

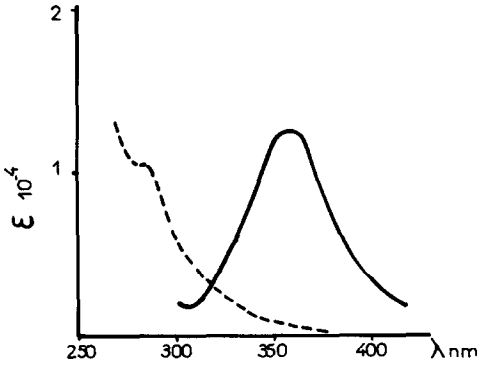


Fig. 1. Absorption spectra of 2-phenyl-4-benzyl-thiazolin-5-one (——) and its hydrolysis product, thiobenzoyl phenylalanine (- - -) at pH 6.6.

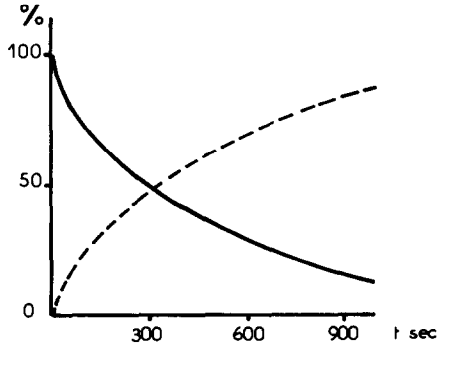
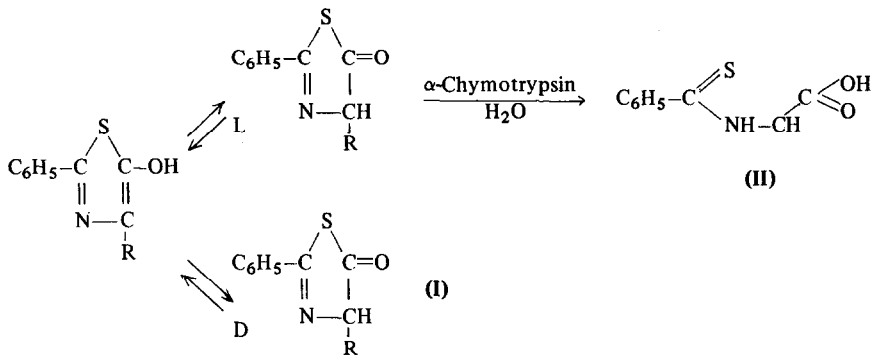


Fig. 2. Decrease of absorbance at 365 nm (——) during hydrolysis of phenyl-DL-phenylalanine-thiazolinone (1×10^{-4} M) catalyzed by α-chymotrypsin (1×10^{-5} M) at pH 6.6 and 25°C. (– – – –) Represents optical rotation of the extracted thiobenzoyl-L-phenylalanine. ($[\alpha]^{25}_{436} = +451$; $c = 0.6$ in methanol).



Since thiazolinones (I) easily racemize while their hydrolysis products (II) are perfectly stable under the utilized conditions, one can suppose that only the L-thiazolinone is hydrolyzed by the enzyme while the D enantiomer does not accumulate but racemizes with a rate which is not a limiting one of the overall process. The non-enzymatic–enzymatic cooperation can be viewed as an invertase system in which the thioacylating group of the substrate acts as a coenzyme for the chymotrypsin.

Another interesting aspect of configuration changes during α-chymotryptic hydrolysis can be observed when the L-MDCOMe is utilized as a substrate. With this molecule, which can be considered a cyclized form of methyl N^{α}acetyl tryptophanate, an inversion of antipodal specificity has been observed, the D enantiomer being much more susceptible than the L one to α-chymotryptic hydrolysis [3].

The D isomer of MDCOMe is a specific substrate for α-chymotrypsin [3] and its hydrolysis yields the expected D-MDC. When L-MDCOMe is utilized as a substrate for α-chymotrypsin a complete inversion of configuration occurs since only the D-MDC can be found at the end point of the reaction.

It should be noted that L- or D-MDCOMe, which can be conserved in optically active form as hydrochloride or dissolved in acidic media, slowly racemize at the pH values utilized for enzymatic hydrolysis while their hydrolysis products are perfectly stable.

The spontaneous racemization constant has been measured as a function of pH between 5 and 9 and reaches a maximum around pH 7 $K'_{pH\,6.8}=9.1\times10^{-4}$. sec^{-1}).

The results reported in fig. 3 show that a continuous increase of enzyme concentration permit the reaction to go beyond the rate of spontaneous racemization until a value of about 10. Since the product of the reaction is always only the D enantiomer of MDC, it can be deduced that the inversion of conformation is catalyzed by the enzyme. This catalyzed isomerisation should precede the hydrolysis of MDCOMe, the hydrolyzed ester being perfectly stable, and constitutes the limiting step in the overall process when the spontaneous racemization is unable to secure a suitable amount of the productive substrate enantiomer.

The two kinds of substrates presented in this work show that the maintaining of configuration is not to be considered as an invariant property of enzymatic re action and could account for some apparent loss or changes in stereospecificity when some elaborate molecules are tested or proposed as substrates.

It will be of certain interest to examine whether configuration changes occur also with other amino

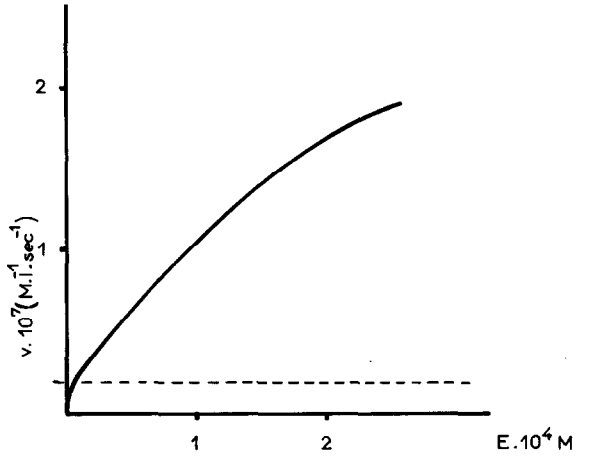


Fig. 3. Rate of hydrolysis of L-MDCOMe as a function of α-chymotrypsin concentration. (- - -) Indicates the non-enzymatic racemization rate.

acid derivatives or with some 'active' intermediates which can be formed during enzymatic reactions and if this phenomenon can be extended to other enzymes.

Furthermore these results are additional examples that the mechanism of an enzyme-catalyzed reaction can be strongly modulated or changed by modification of the substrate molecule [3, 9, 10], and that a clear understanding of the efficiency and specificity of enzyme catalysis in terms of molecular structures must take into account the contribution given by the substrate studied itself.

Acknowledgements

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