INTRAMOLECULAR REACTION OF SPECIFIC SUBSTRATE AS A STEP IN α-CHYMOTRYPSIN CATALYZED HYDROLYSIS*

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1. Introduction

The mechanism of α-chymotrypsin catalyzed hydrolysis of specific substrates is generally represented as follows:

$$E + S \rightleftharpoons [ES] \rightarrow ES' \rightarrow E + P_2 \quad (1)$$
$$\qquad\qquad\quad + \qquad\quad P_1$$

The intermediates [ES] and ES′ are the Michaelis complex and the acylenzyme respectively while P_1 and P_2 are the digestion products. Eq. (1) is also applicable in the low pH region [1]. Nevertheless it has been shown that at least one other important intermediate is formed between the Michaelis complex ([ES]) and the acylenzyme (ES′) [2, 3].

We wish to report the isolation of this intermediate which was identified as an oxazolinone deriving from the intramolecular reaction of the specific substrates. This is in agreement with the influence of the oxazolinone on the hydrolysis of the parent N^{α}acyl amino acid amide when utilized as specific substrate for α-chymotrypsin, as reported in this paper.

2. Materials and methods

Bovine α-chymotrypsin (3X cryst.), FA**–L-tryptophan methyl ester and FA–L-tryptophan were pure commercial products; N^{α}-propionyl-L-tryptophan amide was prepared as described in the routine procedure to obtain acylated amino acid amides [4]; N^{α}-propionyl-L-tryptophan azlactone was prepared from L-tryptophan in propionic anhydride [5]; FA–tryptophan azlactone was obtained through intramolecular dehydration of FA–tryptophan-free acid by the carbodiimide method [6]. Ultraviolet spectra were recorded with a Cary Model 15 spectrophotometer; the autotitrator was a TTT-1 from Radiometer Corp., Copenhagen, Denmark.

2.1. *Enzymatic reactions and analyses of the products*

Stock solutions 5 mM of FA–L-tryptophan derivatives were prepared in acetonitrile. All experiments were performed at 25°. In a typical experiment, 3 ml of α-chymotrypsin solution (0.16 mM) at pH 2.3 were placed in the cuvette of the sample compartment of the spectrophotometer, 0.25 μmoles of the substrate were added and stirred. The change in absorbance at 320 nm was recorded against a reference containing 0.25 μmoles of substrate in 3 ml at pH 2.3 (fig. 1). When analyses of the digestion mixture were necessary the following conditions were employed: to 4 ml of 1.6 mM α-chymotrypsin at pH 2.3, 0.3 ml of acetonitrile, containing 3.3 μmoles of FA–L-Try-O-Me, were added and vigorously stirred. After 90 sec., the reaction was stopped with an excess of acetone at −80°, diluted with cold water and extracted with ethyl acetate in order to analyse for the presence of the substrate or its products. The ethyl acetate layer was carefully dried over Na_2SO_4, taken to dryness under vacuum and the residue chromatographed on silica gel

* Contribution no 41 from the Department.

** *Abbreviations*:
FA- : *N*-3-(2-Furyl)-acryloyl-
TFA: trifluoroacetic acid.

thin layer (Eastman C.S.); the solvent used was chloroform–light petroluem (B.P. 40–60°), 6:4 by vol. (R_f of FA–Try = 0; R_f of FA–Try-O-Me = 0.1; R_f of FA–Try azlactone = 0.3). The direct detection of the oxazolinone derivative was possible by spraying the thin layer chromatogram first with an alcoholic solution of NH_2OH, and after drying at room temperature a few minutes, with an alcoholic solution of $FeCl_3$ and acid dihydrochloride [7]. By this procedure only the oxazolinone derivatives give a positive test: for the ester derivatives longer incubation with NH_2OH at higher temperature is necessary. In a parallel experiment the residue was allowed to react with serine in anhydrous TFA 10 min at room temperature, as previously described [8], as an additional test for the presence of the oxazolinone derivative. The reaction product was chromatographied on Whatman no. 3 paper in 1-butanol–acetic acid–water (4:1:5, by vol.) (R_f of FA–Tyr-O-Ser = 0.80) and revealed by spraying with a solution of ninhydrin or of ferric chloride–potassium ferricyanide [9], a reagent very sensitive for detecting indoles [5]. In all cases parallel runs with known reference products were performed.

N^{α}-Propionyl-tryptophan amide (10 mM) was treated with α-chymotrypsin (0.04 mM) at pH 7,25°. Aliquots of 200 μl were withdrawn from the reaction mixture at suitable intervals and tested with ninhydrin reagent [10] in order to determine the liberated ammonia. Parallel experiments with increasing content of N^{α}-propionyl-L-tryptophan azlactone were performed and the liberation of ammonia, together with the NaOH consumption at the pH-stat, recorded. The first test analyses only the enzymatic digestion of the amide and the second one only the enzymatic digestion of the oxazolinone within the time during which the ninhydrin test is negative.

3. Results

3.1. *Reaction of FA–tryptophan derivatives with α-chymotrypsin at low pH*

The acylation of α-chymotrypsin with specific FA–amino acid esters can be realized in acidic solution and followed with a conventional spectrophotometer. The changes in absorbancy during the reaction, which are at first a rapid increase around 320 nm followed by a slower decrease have been shown in the case of FA–tyrosine-ethylester in a large range of pH [11] and recently in the case of FA–tyrosine-methylester [12] In fig. 1 (curve a) an analogous result is reported for chymotryptic treatment of FA–tryptophan methylester. At the time corresponding to maximum absorbancy the reaction was stopped and, as described under Methods, the organo-soluble material analyzed by thin layer chromatography with inert eluents. The chromatographic analyses showed the presence of a compound indistinguishable from the FA–Try azlactone utilized as a reference compound. The absence of FA–Try proved that no hydrolysis of the substrate had taken place. In a parallel test the organo-soluble material from the enzymatic reaction was treated with serine in anhydrous TFA and FA–Try-O-Ser was obtained. If the enzymatic reaction was stopped after the decrease in absorbance at 320 nm no oxazolinone could be detected.

When FA–Try azlactone is treated with α-chymotrypsin a rapid decrease in absorbance at 320 nm is observed (fig. 1, curve b). The positive peak, observed during the ester hydrolysis, is completely suppressed.

3.2. *Effect of N^{α}-propionyl-L-tryptophan azlactone on the hydrolysis of N^{α}-propionyl-L-tryptophan amide by α-chymotrypsin*

Aqueous solutions of N^{α}-propionyl-L-tryptophan amide (1) containing different amounts of the N^{α}-

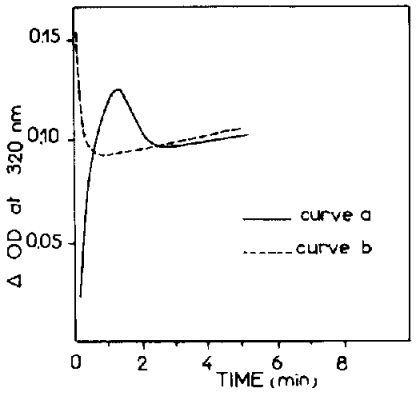


Fig. 1. Change in absorbancy at 320 nm of FA–tryptophan methylester (curve a) and of FA–tryptophan azlactone (curve b) by α-chymotrypsin treatment. pH 2.3, 25°. [E] = 0.16 mM [S] = 0.08 mM.

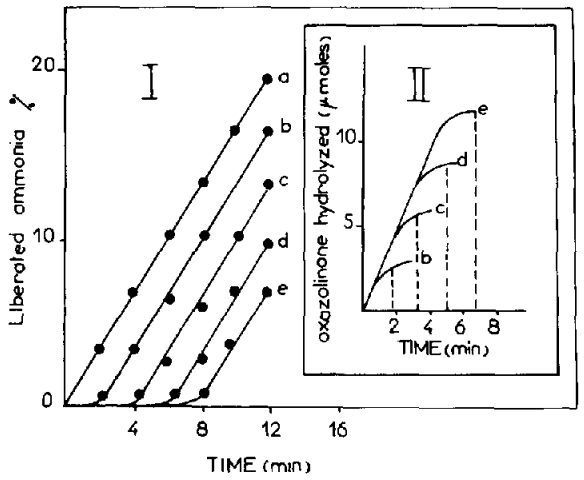


Fig. 2. (I) Liberation of ammonia during the digestion of N^{α}-propionyl-L-tryptophan amide by α-chymotrypsin in presence of N^{α}-propionyl-L-tryptophan azlactone. [E] = 0.04 mM [S] = 10 mM [oxazolinone] = 0 (curve a); 1.2 mM (curve b); 2.4 mM (curve c); 3.6 mM (curve d); 4.8 mM (curve e).

(II) Hydrolysis of different amounts of N^{α}-propionyl-L-tryptophan azlactone by α-chymotrypsin. [E] and [oxazolinone] are the same reported for I b, c, d, and e. pH 7, 25°.

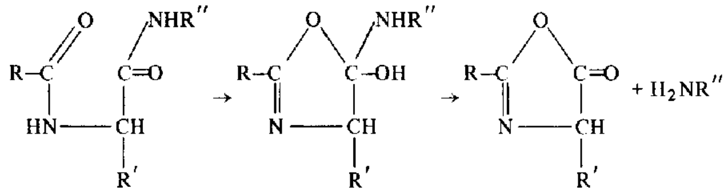
propionyl-L-tryptophan azlactone (III) deriving from its intramolecular reaction as in scheme A (R = ethyl; R′ = indolylmethyl; R″ = H) were treated with α-chymotrypsin at pH 7. The results obtained by parallel NaOH titration and ninhydrin test are summarized in fig. 2. The rate of the chymotryptic catalyzed hydrolysis of the oxazolinone is not affected by the presence of the parent amide substrate while the hydrolysis of the amide starts after a lag time corresponding to a complete hydrolysis of the added oxazolinone. The parallelism of the curves a–e in fig. 2 shows that, after the lag time, the rates of amide hydrolysis are practically unchanged.

N^{α}-Propionyl derivatives were chosen because the N^{α}-propionyl-L-Try azlactone can be easily obtained in its pure optically active form [5].

4. Discussion

The results reported in fig. 1 show that an intermediate is initially formed when a specific chromophoric substrate, as FA–Try-O-Me is treated with α-chymotrypsin. Analogous results and kinetic behaviour have been reported in the case of FA–Tyr-O-Me [12] by Yu and Viswanatha. The chromatographic data reported, strongly suggest that this intermediate is an oxazolinone: the oxazolinone formation can be detected in a large range of pH values but the most suitable conditions for trapping this intermediate are in the low pH region. The spectrophotometric pattern of the enzymatic reaction, the absence of the hydrolysis product of the substrate (FA–Try) at the time corresponding to the maximum absorbance as well as analogous phenomena reported to occur with FA–Tyr-O-Me [12] and FA–Tyr-OEt [11] lead to the conclusion that the formation of the oxazolinone occurs between the Michaelis complex and the acyl-enzyme. This is in agreement with the rapid reaction of oxazolinone with α-chymotrypsin to form the acyl-enzyme [13, 14] while no formation of oxazolinone has been observed during the deacylation of acyl-enzyme [15]. In other words the oxazolinone can be proposed as a highly reactive intermediate in the acylation step of α-chymotrypsin by N^{α}-acylated amino acid derivatives at acidic pH. This point of view seems to find its validity also at neutral pH values as supported by the experiments plotted in fig. 2 in which the behaviour of an oxazolinone derivative is shown to be the one of a highly reactive intermediate during the enzymatic hydrolysis of the corresponding amide substrate rather than the one of a general competitive substrate. However, the generalization of this mechanism to neutral pH requires further direct experimental evidence even though no difference in mechanism as a function of pH has ever been detected in the acylation step of the α-chymotrypsin catalyzed reaction.

In order to correlate these results with the enzymatic mechanism some special features of the chemistry of oxazolinones must be taken into account. It has been shown that N^{α}-acylated amino acids are readily converted to the corresponding oxazolinones when their carboxylic function is activated by a suitable electron-withdrawing substituent [15]. Oxazolinones (III) can also be obtained from N^{α}-acylated amino acid amides (I) through an intramolecular nucleophilic displacement catalyzed by an anhydrous acid solvent [16] as shown in scheme A

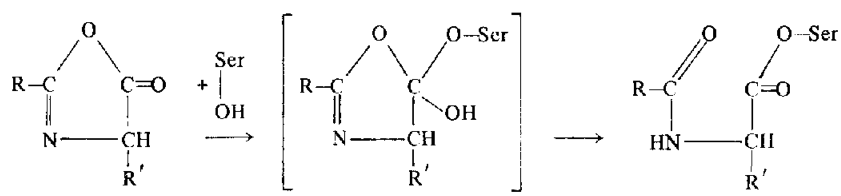


I II III

Scheme A

In this case the electrophilicity of the ultimate carbonyl carbon is enhanced by the solvent protonation. It is noteworthy that in anhydrous acidic medium an N^{α}-acyl amino acid amide (I) easily reacts with serine through the oxazolinone (III) intermediate [8] to yield an N^{α}-acyl amino acyl-O-serine (V) which can be considered as a chemical equivalent of the acylenzyme: (see scheme B)

The closure of a five-membered ring (oxazolinone), which is thermodynamically favoured in non enzymatic reaction [17] should also be favoured in the ES complex. This can account for the fact that the rates of chymotryptic hydrolysis of amino acid esters and amides are markedly increased when these compounds are N^{α}-acylated. The conversion of poor acylating



III IV V

Scheme B

This last reaction has been employed in this work as an additional test to detect the oxazolinone present as an intermediate during the α-chymotryptic hydrolysis of specific substrates as described in experimental part. These organic precedents, together with the oxazolinone formation during the α-chymotryptic hydrolysis of chromophoric specific substrates suggest that the carbonyl group of the susceptible bond could be activated in the enzyme–substrate complex and therefore attacked by the carbonyl oxygen of the acyl group. The first intermediate formed should be an internal tetrahedral intermediate (II) which subsequently gives the oxazolinone with the splitting of the first product (P_1 in eq. 1). In any case the possibility of postulating the oxazolinone as an intermediate ES* formed before the acylenzyme arises from our results and can be written as shown below:

$$E + S \rightleftharpoons [ES] \rightarrow ES^* \rightarrow ES' \rightarrow P + P_2$$
$$+ P_1$$

agents such as amino acid amides into strong acylating agents such as oxazolinones can clarify the controversy existing in the literature over the general validity of the acylenzyme hypothesis even for the natural substrates of α-chymotrypsin. Furthermore the presence of the oxazolinone in the digestion mixture can account for some secondary reactions which seem to exclude the acylenzyme intermediate under special experimental conditions [18–20]. If one supposes that this proposed mechanism is not relative to a secondary or alternative pathway of the enzymatic reaction but to the principal one, the formation of the acylenzyme is unnecessary for the lytic process *per se* and could have a different function, conceivably one of regulating the rate of hydrolysis, since the cleavage of the susceptible bond could be achieved before the acylenzyme formation.

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