INTRAMOLECULAR DEHYDRATATION OF PHENYLTHIOCARBAMYL AMINOACIDS INDUCED BY α-CHYMOTRYPSIN*

M.-A. COLETTI-PREVIERO, C. AXELRUD-CAVADORE and A. PREVIERO

Département de Biochimie Macromoléculaire (CNRS) and Groupe de Recherches sur la Pathologie Cellulaire et Moléculaire du Globule Rouge (INSERM), B.P. 1018, 34-Montpellier, France

Received 18 October 1970

1. Introduction

In a previous paper [1] evidence was presented that α-chymotrypsin initially induces an intramolecular reaction of specific substrates (acyl amino acid ester or amide) to yield an oxazolinone. The general mechanism of the enzymatic hydrolysis was therefore represented as follows:

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

where [ES] is the Michaelis complex, ES* the enzyme-oxazolinone complex and ES′ the acylenzyme. P_1 and P_2 are the digestion products.

The ability of α-chymotrypsin to induce the cyclisation of N^{α}-substituted amino acid derivatives seems to be a general one as demonstrated in this paper by the enzyme catalyzed conversion of PTC** derivatives of aromatic amino acids to their corresponding PTH.

The idea that functional groups responsible for the enzymatic catalysis are in part to be found in the substrate itself in the specially organized enzyme–substrate complex is discussed.

2. Materials and methods

Bovine α-chromotrypsin (3 × cryst.) was purchased from Worthington Biochemical Corp., Freehold, N.J.; PTC and PTH derivatives were obtained from the parent amino acids and phenylisothiocyanate by the usual techniques [2].

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*

PTC derivative (0.21 mM) was incubated with α-chymotrypsin (0.016 mM) at different pH values. The extent of the PTC → PTH conversion was followed by differential spectrophotometry [3]. In the reference double cell the enzyme and the PTC derivative were each in one compartment, in the sample double cell they were in the same compartment and buffer in the other. Differential spectra obtained with calibrated solutions of PTC and PTH amino acids were utilized to calculate the extent of the reaction.

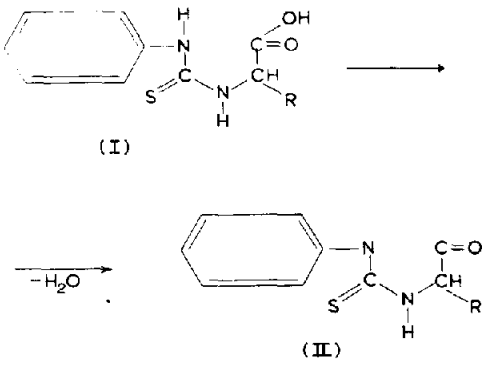
3. Results

The spectral changes following the conversion of PTC amino acids (I) to the corresponding PTH (II) are essentially an increase of the absorbancy at 265 nm and a sharp decrease at 241 nm [4]. In fig. 1 the difference spectra observed during the α-chymotrypsin treatment of PTC–L-Try at pH 4.6 are recorded. A chromatographic control showed that only the PTH derivative was present at the end-point of the enzymatic reaction.

* Contribution no. 42 from the Department.

** *Abbreviations*: PTC: phenylthiocarbamyl; PTH: phenylthiohydantoine.

North-Holland Publishing Company – Amsterdam



The pH dependence of PTC → PTH conversion catalyzed by α-chymotrypsin is reported in fig. 2 for the L-tryptophan derivative (*curve a*). Analogous spectral changes and pH depending rates were obtained when the PTC derivatives of L-Tyr (*curve b*) and L-Phe (*curve c*) were treated with α-chymotrypsin under the conditions reported for the tryptophan derivative. The ability of α-chymotrypsin to easily induce the cyclisation of the PTC amino acids is restricted to the L-Try, L-Tyr and L-Phe derivatives. The D-isomers of these PTC aromatic amino acids as well as the PTC of other amino acids are not transformed by α-chymotrypsin as shown in table 1.

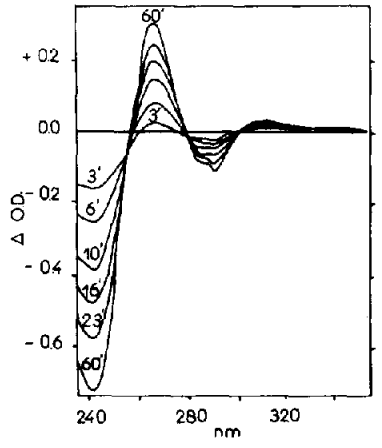


Fig. 1. Difference spectra during the α-chymotrypsin (0.016 mM) treatment of PTC-L-tryptophan (0.21 mM) at pH 4.6, 25°.

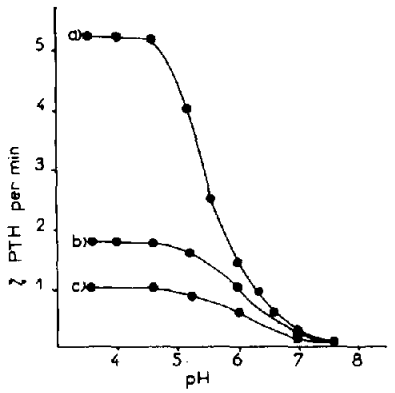


Fig. 2. Initial velocity of α-chymotryptic catalyzed PTC → PTH conversion as a function of pH. PTC-L-tryptophan (*curve a*); PTC-L-tyrosine (*curve b*); PTC-L-phenylalanine (*curve c*). [E] = 0.016 mM [S] = 0.21 mM.

Table 1

Substrate PTC–	% PTH in 30 min.
– L-Try	100 (50 in 2 min)
– L-Tyr	49.2
– L-Phe	25.4
– D-Try – D-Tyr – D-Phe	0
– Gly – L-Ala – L-Val – L-Arg	0

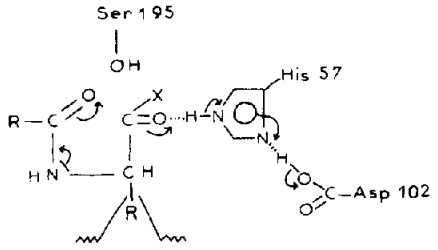
Dehydratation of PTC derivatives (0.21 mM) promoted by α-chymotropsin (0.08 mM), pH 4.6, 25°.

4. Discussion

The intramolecular reaction of specific substrates induced by α-chymotrypsin yields an oxazolinone [1] which is a strong acylating agent for the "active" serine of the enzyme [5, 6]. Therefore its presence in the digestion mixture can be detected only under special experimental conditions and its accumulation is a function of its rate of formation and of the rate of its consecutive reaction to acylenzyme. On the other hand it has been demonstrated that specific N^{α}-acyl amino acid free acids (called "virtual substrates") are

able to form the acylenzyme with α-chymotrypsin [7] through a mechanism which should be analogous to the one of true substrates. We have considered the PTC derivatives of L-aromatic amino acids as virtual substrates for α-chymotrypsin. In this case, the expected cyclic intermediate (S* in eq. 1) deriving from the intramolecular reaction of the substrate induced by the enzyme is a non-acylating agent, the PTH-derivative, which constitutes the final product of the enzymatic reaction. In this special case the chymotryptic catalysis should stop before the acylenzyme so that the hydrolytic enzyme catalyses only a dehydratation reaction. This proved to be the case, as depicted in fig. 1 and quantitatively determined in table 1. The spectral evidence and the chromatographic controls [4] showed that only PTH is formed without any formation of isomeric thioazolinone (λ_{max} = 252 nm, λ_{min} = 225 nm), which is currently encountered in chemical cyclisation of PTC amino acid derivatives. The specificity of α-chymotrypsin in these enzymatic dehydratations is not altered as shown by the inertness of the D-isomers of aromatic amino acids and of other PTC derivatives (table 1). The kind of reaction (a dehydratation) together with the pH profile of PTC → PTH enzymatic conversion (fig. 2) strongly suggests that the acylenzyme intermediate should not be concerned [8].

The structure of the complex of α-chymotrypsin with its virtual substrates, recently investigated [9], allowed the authors to suggest that the susceptible bond of a true substrate is near to the histidine 57 and the serine 195 and that this could account for the mechanism of catalysis proposed by Blow et al. [10]. This, together with the information gained by the detection of a new intermediate during the hydrolysis of specific substrates [1] as well as the intramolecular dehydratation of PTC to PTH induced by α-chymotrypsin allows one to propose a tentative mechanism which could account for the intramolecular reaction of specific substrates induced by the enzyme.



In this tentative mechanism we wish to point out that the carbonyl carbon of the susceptible bond (or the carboxyl in virtual substrates), activated in the enzyme–substrate complex, can be attacked either in termolecularly by the "active" serine, or intramolecularly by a suitable nucleophile which is the N^{α}-acyl group, when N^{α}-acylated amino acid amides and esters are concerned and the thiocarbamyl group in the case of PTC amino acids. One can suppose that substrates such as *p*-nitrophenylacetate and some amino acid esters which are true acylating agents can directly acylate the "active" serine in the ES complex even if they are unable to undergo intramolecular cyclisation. It can be seen that the mechanism, at least in the chymotryptic hydrolysis, seems to be dependent on the choice of the substrate studied.

It does not escape our attention that the intramolecular reaction, observed in the α-chymotrypsin catalyzed hydrolysis of specific substrates could constitute a general step for other lytic enzymes. Some preliminary results obtained in our laboratory seem to confirm this hypothesis.

Acknowledgements

We wish to express our sincere gratitude to Dr.E. Zuckerkandl for his stimulating advice, encouragement and discussion throughout this research and Mrs. Denise Waeckerle for skillful technical assistance.

References

[1] M.-A. Coletti-Previero, C. Axelrud-Cavadore and A. Previero, FEBS Letters 11 (1970) 213.
[2] D. Ilse and P. Edman, Australian J. Chem. 16 (1963) 411.
[3] T. Herskovits and M. Laskowski, J. Biol. Chem. 237 (1962) 2481.
[4] P. Edman, Acta Chem. Scand. 10 (1956) 761.
[5] J. De Jersey and B. Zerner, Biochemistry 8 (1969) 1967.
[6] A. Previero and M.-A. Coletti-Previero, Compt. Rend. Acad. Sci. 268 (1969) 1798.
[7] F.J. Kezdy, G.E. Clement and M.L. Bender, J. Am. Chem. Soc. 86 (1964) 3690.
[8] M.L. Bender and F.J. Kezdy, J. Am. Chem. Soc. 86 (1964) 3704.
[9] T.A. Steitz, R. Henderson and D.M. Blow, J. Mol. Biol. 46 (1969) 337.
[10] D.M. Blow, J.J. Birktaft and B.S. Hartley, Nature 221 (1969) 337.