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Formation and Cleavage of Thiohydantoin Ring Catalysed by Trypsin

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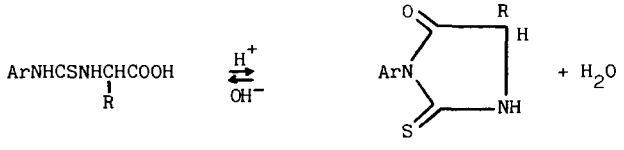
The intramolecular dehydratation of phenyl thiocarbamyl amino acid to the corresponding thiohydantoin and the reverse hydrolytic reaction are specifically and separately catalyzed by trypsin at two different pH values when L-Arg derivatives are concerned. These substrates disclose the ability of trypsin to catalyze unexpected synthetic and hydrolytic reactions.

The direct participation of the substrate molecule in the mechanism of enzymatic action has been suggested on the basis of some experimental evidence involving trypsin (*1*) and α-chymotrypsin (*2*). The basic idea was that the functional groups involved in the enzymatic catalysis are localized not only in the enzyme molecule but, to a certain extent, also in the substrate moiety of the enzyme–substrate complex. The immediate consequence is that a change in the number or in the nature of "active" functional groups in the enzyme–substrate complex can be achieved using suitable compounds as substrates in which the desired functional groups are linked to the specific structure capable of specific interaction with the enzyme (*3*).

Enzymatic specificity, usually defined on the basis of the range of compounds which are substrates for a given enzyme (*4*), can thus include a large number of unexpected chemical reactions, unexpressed with natural or natural-like compounds (*5, 6*).

We report in this paper on the ability of trypsin to catalyze either the synthesis or the hydrolysis of thiohydantoins in two separate pH regions. Thiohydantoins are formed by acid-catalyzed cyclization of thiocarbamyl amino acids and the reaction can be reversed by alkaline hydrolysis.



For Arg derivative: R = $\cdot CH_2CH_2CH_2NH(NH_2):NH$.

The cyclization of phenyl thiocarbamyl arginine (PTC) and the hydrolysis of the

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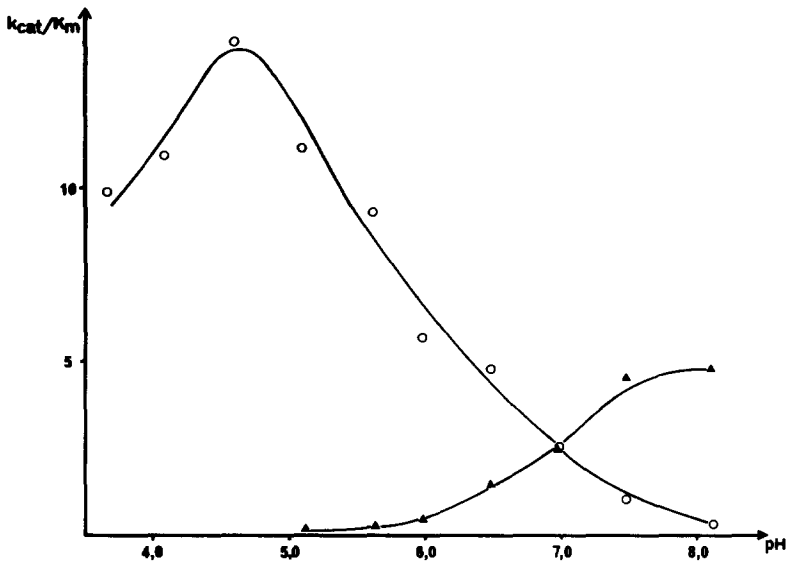


FIG. 1. Kinetic parameters of trypsin-catalyzed cyclization PTC-L-Arg → PTH Arg (O) and PTH Arg → PTC-L-Arg hydrolysis (▲) plotted against pH. Temperature = 25°C. Rate constant in $sec^{-1} M^{-1}$ units. The pK_a value of PTC Arg from the rising part of the curve (O) is 4.1.

obtained phenyl thiohydantoin (PTH) are reactions the rates of which depend on different factors such as pH, ring size, and type of ring substituents (*7*). The ring cleavage is a pseudomonomolecular reaction which differs from the ring closure reaction and cannot be compared with it because the guanido-alkyl ring substituent is in the nonprotonated form. Furthermore the cyclization of PTC amino acids takes place through a thiazolinone intermediate (*8*) which is not necessarily formed during the alkaline hydrolysis of PTC. These two reactions have been separately studied in the presence of trypsin. The intramolecular dehydration of PTC-L-arginine is catalyzed by trypsin (Fig. 1). The Michaelis–Menten parameters plotted against pH show an optimum trypsin catalysis around pH 4.5 and their order of magnitude lies between those of nonspecific and specific substrates. The specificity of trypsin for PTC-L-Arg results also from the comparison with PTC-D-Arg and PTC derivatives of the common neutral amino acids which are practically inert toward trypsin catalysis under the same experimental conditions. The trypsin catalysis in the hydrolysis of PTH-Arg appears at pH values near neutrality and increases until about pH 8. The PTC-L-Arg and PTH-Arg are then considered either substrates or enzymatic reaction products for trypsin when acidic or basic pH are respectively considered.

From a steric point of view it should be outlined that solutions of PTH Arg are optically inactive, probably because a tautomeric equilibrium rapidly induces racemization of the two enantiomers:

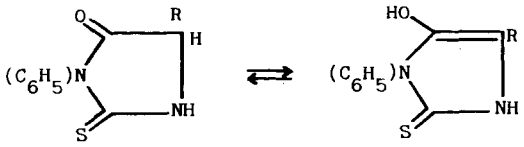


TABLE 1

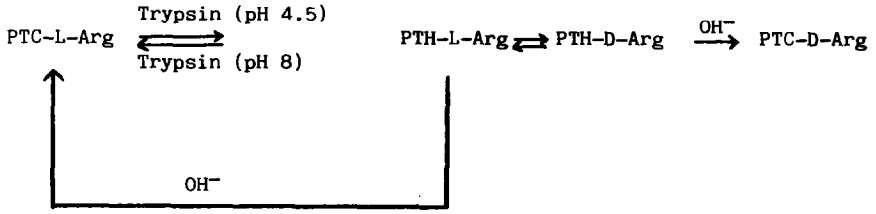
INCREASE IN OPTICAL ACTIVITY DURING TRYPSIN-CATALYZED HYDROLYSIS OF PTH ARG[a]

t (min)	$\Delta\alpha^{0}_{436}$
0	0.000
1	+0.011
7	+0.022
13	+0.025
30	+0.032
60	+0.039

[a] C = 0.2 in 0.1 M phosphate buffer, pH 8; trypsin concentration = 10^{-4} M; temperature = 25°C.

The tryptic hydrolysis of PTH-DL-Arg gives only the optically active PTC-L-Arg directly from the L form of PTH and indirectly from the PTH-D-Arg through its racemization, and the reaction can be followed by the appearance of the optical activity (Table 1).

The enzymatic interconversion PTC-L-Arg ⇄ PTH Arg can be performed several times through a pH oscillation between the pH values optimum for the two reactions, even if a progressive accumulation of nonproductive PTC-D-Arg takes place through the nonenzymatic hydrolysis of the D form of PTH Arg.



EXPERIMENTAL

Materials and Methods

Bovine trypsin and PTH-L-Arg (λ_{max} = 265 nm, log ϵ = 4.168 in water) were pure commercial products. PTC-L-Arg was obtained by reacting L-Arg with phenylisothiocyanate (*9*) as follows. L-Arg monohydrochloride (0.210 g) was dissolved in 0.1 N $NaHCO_3$–Na_2CO_3 buffer at pH 8.9 (10 ml) and treated with phenylisothiocyanate (0.135 g) dissolved in methanol (10 ml) at 40°C. An apparent pH value of 8.9 was maintained by addition of 1 N NaOH during 30 min. After cooling at 0°C, the sodium salt of PTC-L-Arg crystallizes as needles: yield 0.210 g;

mp 180°C d; log ϵ (λ_{max}) 4.143 (245 nm) in water; $[\alpha]_{589}^{20} = +18.6$ and $[\alpha]_{436}^{20} = +41.2$; $C = 1$ in methanol.

Anal. Calcd for $C_{13}H_{18}O_2N_5NaS \cdot HCl$: C, 42.44; H, 4.93; N, 19.04; S, 8.71. Found: C, 42.91; H, 5.01; N, 18.97; S, 8.92.

Optical activities were measured using a Roussel Jouan micropolarimeter Type 71. PTC and PTH Arg obtained after the enzymatic reaction were analyzed by thin-layer chromatography (*8, 10*) and identified by comparison with authentic samples. Spectral behavior of the reaction mixtures was identical to that of the mixed products.

Kinetic Measurements

Trypsin-catalyzed cyclization PTC Arg → PTH Arg as well as hydrolysis of the PTH derivative were measured in 0.1 *M* acetate buffer (in the region $3.6 \leq pH \leq 5.6$) and 0.1 *M* phosphate buffer (in the region $5.7 \leq pH \leq 8.1$) using a Beckman spectrophotometer Acta C III with cell compartments at 25°C. The type of buffer did not influence the extent of the reaction. Extent of formation and hydrolysis of PTH was followed by the absorbance changes (ΔA) at 245 and 265 nm. The reaction rates were measured with the initial velocity approximation by measuring ΔA values per unit time. The rate expression

$$\nu = \frac{k_{cat}}{K_m} E_0 S$$

was followed by both PTC → PTH and PTH → PTC enzymatic conversions in the pH range of Fig. 1: ν represents the reaction rate at time t (sec), k_{cat} and K_m the usual Michaelis–Menten parameters, [S] the substrate concentration at time t, and $[E_0]$ the starting enzyme concentration. Maximum concentration utilized was 10^{-4} *M* for the substrate and 5×10^{-6} for trypsin. The nonenzymatic rate value was always subtracted when enzymatic reactions were considered. At pH values where an equilibrium mixture of both PTC and PTH was present, the k_{obs} value was obviously the sum of forward and reverse rate constants.

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