THE INHIBITION OF TRYPSIN, PLASMIN, AND THROMBIN BY BENZYL 4-GUANIDINOBENZOATE AND 4'-NITROBENZYL 4-GUANIDINOBENZOATE

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The inhibition of the enzymes trypsin, plasmin, and thrombin by benzyl 4-guanidinobenzoate and 4'-nitrobenzyl 4-guanidinobenzoate is caused by acylation of the active site. Second order rate constants were determined.

1. Introduction

Recently benzyl 4-guanidinobenzoate (BGB) and 4'-nitrobenzyl 4-guanidinobenzoate (NBGB) were described as very potent competitive inhibitors of trypsin [1]. Since other esters of 4-guanidinobenzoic acid are known to inhibit the enzyme by forming covalent bonds [2–4], the mode of action of BGB and NBGB was reinvestigated. Furthermore, the action of these compounds against plasmin and thrombin was studied.

2. Materials and methods

2.1. *Inhibitors, enzymes, and substrates*

Benzyl 4-guanidinobenzoate (BGB). HNO_3, 4'-nitrobenzyl 4-guanidinobenzoate (NBGB). HNO_3, and 4-nitrobenzyl alcohol were kindly supplied by Prof. Dr. G.Wagner, Sektion Biowissenschaften, Karl-Marx-Universität Leipzig. Trypsin (E.C. 3.4.4.4), a crystallized preparation from bovine pancreas (Spofa, Prague) containing approximately 50 percent magnesium sulphate, plasmin (E.C. 3.4.4.14) was obtained from human plasminogen prepared according to Wallén [5], specific activity: 40 caseinolytic units per mg nitrogen by activation with streptokinase at pH 7.2 (0.35 units of streptokinase per caseinolytic unit of plasminogen). Thrombin (E.C. 3.4.4.13) prepared from bovine blood according to Walsmann [6], specific activity 1300 NIH-units per mg. Streptokinase, Streptase® from Behring Werke AG, Marburg/Lahn. Fibrinogen, a bovine preparation from Behring Werke AG, Marburg/Lahn. *N*-α-Benzoyl-DL-arginine-4-nitroanilide. HCl (BANI), Fluka AG, Buchs, Switzerland.

2.2. *Inactivation of trypsin, plasmin, and thrombin*

Trypsin (40 μg/ml), plasmin (14 caseinolytic units/ml), or thrombin (50 NIH-units/ml) were incubated at 25° with suitable concentrations of BGB or NBGB. All reactants were dissolved in 0.1 M tris-HCl buffer containing 0.05 M NaCl and 0.01 M $CaCl_2$, pH 7.2. At different times, samples of the incubation mixture were taken and the reaction between enzyme and inhibitor was stopped by addition of substrate (BANI). The residual activity of the enzymes was measured by determining the hydrolysis of BANI (0.002 M) at pH 7.2 and 25° as described previously [7].

3. Results

3.1. *Inhibition of trypsin*

By incubation of trypsin with one of the guanidinobenzoic acid esters, an inhibition of the enzyme is evident. The extent of the inhibition is dependent on the concentration of the ester and the incubation time. The reaction shows second order kinetics as can be seen from a plot of log $(\frac{a-x}{b-x})$ against the reaction time (fig. 1). Second order rate constants were determined graphically from the slope of the resulting lines as a parameter of the inhibitory activity (table 1).

According to the kinetics described for trypsin inactivation by both the esters, formation of a covalent

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Table 1
Rate constants for the reaction of benzyl 4-guanidinobenzoate (BGB) and 4′-nitrobenzyl 4-guanidinobenzoate (NBGB) with trypsin, plasmin, and thrombin at pH 7.2.

Inhibitors	k^* ($M^{-1} \cdot sec^{-1}$)		
	Trypsin	Plasmin	Thrombin
Benzyl 4-guanidinobenzoate	930	20	0.9
4′-Nitrobenzyl 4-guanidinobenzoate	2500	70	1.1

* Second order rate constant.

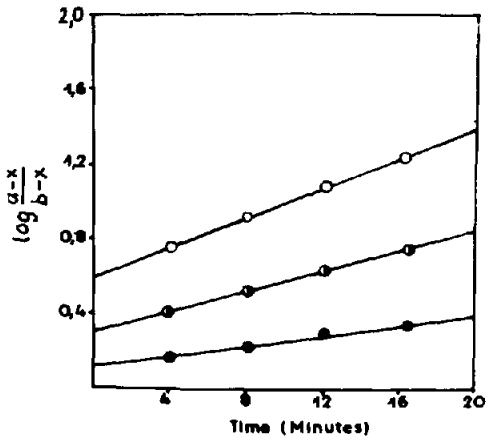

Fig. 1. Inactivation of trypsin (8.4×10^{-7} M) by 4′-guanidinobenzoate (○ = 2.1×10^{-7} M, ◑ = 4.2×10^{-7} M, ● = 6.3×10^{-7} M). (b = starting concentration of the inhibitor, a = starting concentration of the enzyme, x = quantity of enzyme inactivated at time t).

bond is supposed. Evidently, the inhibitors react like a substrate with trypsin and hydrolysis stops at the step of acylenzyme. The reaction of the inhibitors results in the formation of 4-guanidinobenzoic acid ester of trypsin (transesterification). Therefore, only one split product (benzylalcohol or 4-nitrobenzylalcohol) can appear and the number of ester bonds must remain constant during the inactivation.

For demonstrating the occurrence of this split product, equimolar amounts (10^{-3} M) of trypsin and 4′-nitrobenzyl 4-guanidinobenzoate were incubated at pH 7.2 and 25° for 10 min. After the incubation,

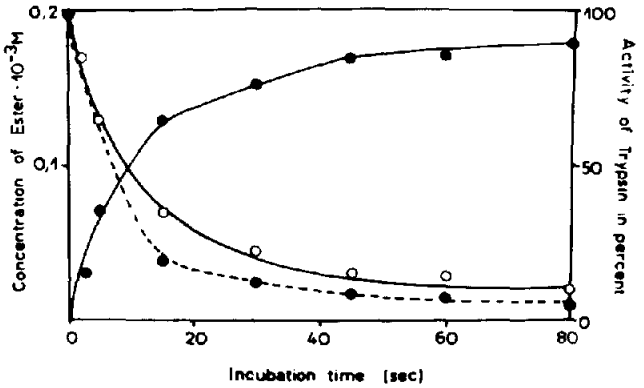

Fig. 2. Transesterification during the inactivation of trypsin by equimolar amounts of 4′-nitrobenzyl 4-guanidinobenzoate. Ester concentration in the trypsin-free supernatant (○) and in the trypsin precipitate suspended in equal volumes of water (●). Trypsin activity (- - -).

benzyl alcohol and 4-nitrobenzyl alcohol were detected by means of thin layer chromatography. In order to demonstrate the transesterification, equal volumes of 4×10^{-4} M solutions of trypsin and 4′-nitrobenzyl 4-guanidinobenzoate were incubated at pH 7.2 at 25°. Samples were taken at different times and mixed with equal volumes of 6 percent perchloric acid. The precipitate was centrifuged. The concentration of ester in the supernatant and in the precipitate was determined by the hydroxamic acid method [8]. The time-dependent decrease of the ester concentration in the trypsin-free supernatant corresponds with an increase of the ester content in the trypsin precipitate (fig. 2).

3.3. *Inhibition of plasmin and thrombin*

NBGB and BGB inhibit not only trypsin but also plasmin and thrombin. The inhibition of plasmin and thrombin takes place in the same manner, as in the case of trypsin. In order to evaluate the velocity of the inactivation, the second order rate constants of the inhibition of plasmin and thrombin were determined at pH 7.2 in a similar manner, as described above (table 1). First order kinetics were applied because an important excess of inhibitor was required for the inactivation of plasmin and thrombin during the investigated time interval. Consequently, plasmin and thrombin are inactivated much more slowly than trypsin.

4. Discussion

The result that BGB and NBGB are stronger inhibitors of trypsin than benzyl 4-amidinobenzoate was surprising. In other investigations [1, 7, 9, 10] benzamidin compounds were accordingly found to be more potent competitive inhibitors of trypsin than the corresponding derivatives of phenylguanidine. Our investigations on the mechanism of the trypsin inactivation by benzyl 4-guanidinobenzoate and 4′-nitrobenzyl 4-guanidinobenzoate elucidate the especially strong inhibition of trypsin by these compounds. The strong inhibition is not caused by a competitive mechanism but by acylation of the enzyme by these esters. The deacylation takes place so slowly that a practically irreversible inhibition results. The inactivation runs relatively rapidly in comparison with other irreversible inhibitors of trypsin (TLCK [11], DFP [12], diphenyl carbamyl chloride [13], 4-guanidinobenzoic acid ethyl ester [4]).

Plasmin and thrombin are inhibited in the same way. However, the plasmin inactivation is 30–50 times slower. The inactivation of thrombin was found to be about 1000 times lower. Similar relations of the rates for the inactivation of trypsin, plasmin, and thrombin were found by Chase and Shaw [3].

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