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THE KINETICS OF REACTION OF BOVINE THROMBIN WITH p-NITROPHENYL p'-GUANIDINOBENZOATE AND 7-AMINO-1-CHLORO-3-TOLUENE-p-SULPHONAMIDO-2-BUTANONE: A NEW METHOD FOR DETERMINING THE OPERATIONAL MOLARITY OF THROMBIN SOLUTIONS

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1. Introduction

New reagents for determining the operational molarities of solutions of bovine trypsin and chymotrypsin have been developed recently in this laboratory [1, 2]. These and several other compounds were found not to be suitable for titrating solutions of bovine thrombin. We have now found that *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB), which has been synthesized recently and used as a titrant for trypsin by Chase and Shaw [3], is an excellent reagent for determining the operational molarity of solutions of bovine thrombin. A stoichiometric amount of *p*-nitrophenol is rapidly liberated by the enzyme. The zero-order constants, k_0, for the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE) by thrombin and trypsin are similar in magnitude. The rate of acylation of thrombin by NPGB has been determined by the stopped-flow technique. The kinetics of inhibition of thrombin by 7-amino-1-chloro-3-toluene-*p*-sulphonamido-2-butanone (TLCK) have been determined using NPGB to titrate residual enzyme activity in aliquots of reaction mixture removed at intervals of time.

2. Experimental

Bovine thrombin was either a gift from Dr. S. Magnusson or was prepared by his method [4, 5]; solutions of thrombin were centrifuged at 20000 g for 10 min before use. Human plasmin, obtained by glycerol activation of plasminogen, was a gift from Dr. P. Wallén. NPGB was synthesized by the method of Chase and Shaw [3]; stock solutions in *N*-methylpyrrolid-2-one were not stored for longer than two days. Measurements of light absorption were made using an Optica CF 4 single-beam spectrophotometer with a ¼ sec response Honeywell recorder. The kinetics of reaction between NPGB and thrombin were followed by the stopped-flow technique; the apparatus of Gibson and Milnes [6] was attached to the spectrophotometer [1] and the progress of the reaction was displayed on a Tektronix 564 storage oscilloscope.

2.1. *Determination of the operational molarity of thrombin solutions*

Thrombin solution (0.15–1.50 mg in 3 ml) in 0.1 M-sodium barbiturate buffer, pH 8.3, was placed in a cuvette of 50 mm light path at 25° in the spectrophotometer. Buffer (3 ml) was placed in the reference cuvette. 5 mM-NPGB in *N*-methylpyrrolid-2-one (0.03 ml) was added to each cell, the solutions were mixed, cell lids were replaced and the light extinction was measured at 410 mμ within 30 sec of addition of ester. The amount of thrombin present was calculated from the concentration of *p*-nitrophenol produced ($\epsilon_{410m\mu}$ = 16 595 at pH 8.3 and 25° [3]). No turnover due to thrombin-catalysed hydrolysis was detected, but NPGB was slowly hydrolysed non-enzymatically. The burst of *p*-nitrophenol was shown in separate experiments to be independent of substrate concentration in the range 16.6–82.0

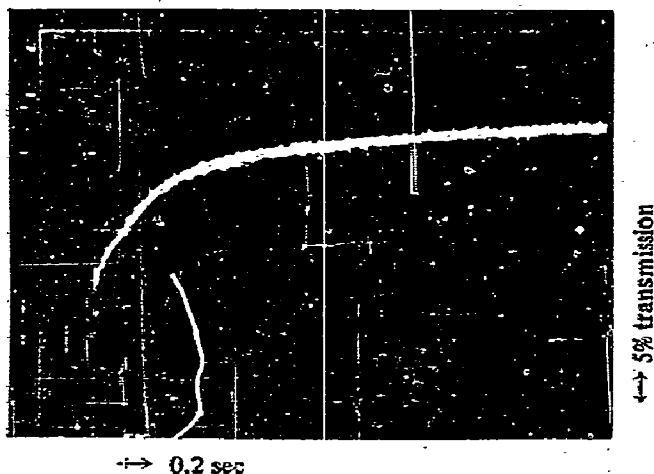

Fig. 1. Progress of the reaction between NPGB and thrombin at pH 8.3 and 25° followed at 410 mμ.

μM and to be proportional to thrombin concentration in the range 0.3–7.6 μM. After reaction with NPGB, clotting and esterase activities of thrombin were destroyed.

Using a standardized thrombin solution, k_0 for the hydrolysis of BAEE by thrombin using a pH-stat was found to be 12.5 ± 0.9 sec^{-1} at pH 8.4 and 25° in the presence of 0.1 M sodium chloride.

2.2. *Kinetics of reaction of NPGB with thrombin*

A 0.2 mM solution of NPGB in *N*-methylpyrrolid-2-one — water (1:49) was prepared by dilution of a 10 mM stock solution in *N*-methylpyrrolid-2-one. Equal volumes of the diluted ester solution and of thrombin (0.5 mg/ml) in 5 mM sodium barbiturate solution, pH 8.3, were mixed at 25° by the stopped-flow technique and the change in percentage transmission of light at 410 mμ was recorded oscilloscopically (fig. 1). The regression curve of $\log_{10}(E' - E)$ on t, where E'_i and E_i are extinction values at times t_i and $t_i + \tau$ [7], was calculated by the least squares method and the first-order rate constant was found to be 4.05 ± 0.10 sec^{-1}.

2.3. *Kinetics of reaction of TLCK with thrombin*

Thrombin (10.6 μM) and TLCK (2.41 mM) were allowed to react at 20° in 0.1 M sodium barbiturate buffer at pH 8.3. Aliquots were withdrawn at intervals and residual active thrombin was titrated with NPGB. First-order kinetics ($k \approx 4.4 \times 10^{-3}$ sec^{-1}) were observed for at least 75% of the reaction; slight deviations therefore were probably due to competing hydrolysis of TLCK, but complete inhibition of reaction towards NPGB and of esterase and clotting activities was achieved. The velocity at zero time was computed by a curve-fitting procedure and the second-order rate constant was found to be 1.34 ± 0.05 M^{-1} sec^{-1}.

3. Discussion

NPGB fulfils the requirements [1, 8] of a specific titrant of thrombin. The enzyme is acylated rapidly and the rate of deacylation is negligible within the time required to measure the 'burst' of *p*-nitrophenol. Approximately 50% of the enzyme was found to be active based on a molecular weight of 33 700 [9]. Although the percentage purity is apparently low, there is evidence [10, 11] that thrombin binds peptides rather firmly. With a standardized solution of thrombin, the value of k_0 for the hydrolysis of BAEE was found to be very similar to the values reported for human thrombin [12] and bovine trypsin [8].

The reaction of TLCK with thrombin appears to be somewhat slower than the corresponding inhibition of trypsin. Shaw, Mares-Guia and Cohen [13] report a second-order rate constant of 5.6 $M^{-1}sec^{-1}$ for the latter reaction at pH 6.0 and 25°. This rate constant was about five times greater at pH 8.0. Although it is not known if TLCK reacts with a histidine residue at the active centre of thrombin analogous to the inhibition of trypsin, the present work indicates that TLCK and NPGB react at the same or overlapping sites in thrombin. That this site is the active centre follows from our observation that the interaction of TLCK or NPGB with thrombin completely inhibits clotting and esterase (BAEE) activities.

Plasmin, derived from human plasminogen by activation in 50% glycerol, did not liberate *p*-nitrophenol from NPGB. In contrast, Chase and Shaw [14] have found that plasmin produced by urokinase activation of human plasminogen, is acylated by NPGB. These observations presumably reflect a significant difference in structure of the plasmin which stems from the two methods of activation of the zymogen.

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