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INHIBITION OF ESTERASE AND AMIDASE ACTIVITIES OF α- AND β-THROMBIN IN THE PRESENCE OF ANTITHROMBIN III AND HEPARIN

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Summary

Inhibition of the esterase and amidase activities of bovine α- and β-thrombin in the presence of antithrombin III and heparin has been studied. It was found that both the esterase and amidase activities of α-thrombin were inhibited by antithrombin III and the reactions were accelerated by heparin. The inhibition of amidase and esterase activities of β-thrombin by antithrombin III has also been demonstrated. Heparin however did not increase the rate of inactivation of the enzyme.

Introduction

One of the main functions of the enzyme thrombin is to initiate the conversion of its natural soluble substrate fibrinogen to the insoluble product, fibrin [1]. Besides the above action, thrombin readily splits various synthetic substrates (arginine and lysine peptides and esters) similarly to trypsin [2—5].

In the past few years multiple active forms of bovine as well as human thrombin have been observed [6—19], although they were derived from the "zymogen" under various activation procedures. It has been proved, that bovine thrombin consists of at least three form, with molecular weights of 39 000 (α-thrombin), 28 000 (β-thrombin) and 28 000 (γ-thrombin). The nomenclature is based on their order of formation from prothrombin and is also a reflection of the strength of their adsorption to the sulphopropyl-Sephadex column, what is used for the separation. β-Thrombin is formed from α-thrombin by degradation of the A chain and excision of a small, carbohydrate containing fragment. The three major forms of bovine thrombin were found to possess different relative activities toward fibrinogen and small peptide substrates, but had similar esterase activities [12,13,17,19]. We have

demonstrated that α- and β-thrombin differed not only in their clotting activity, but also in their heparin sensitivity [20]. There were also differences in their inactivation by antithrombin III or α-1-proteinase inhibitor [21].

It is accepted that the activity as well as the formation of thrombin during blood coagulation is regulated by several inhibitors. Among them antithrombin III (heparin cofactor), with a molecular weight of 65 000 accounts for the large part of the 'progressive' antithrombin activity of normal plasma and serum [22]. The reaction between thrombin and antithrombin III exhibits 1 : 1 stoiechiometry and the rate of inhibition is markedly accelerated by small amounts of heparin [23,24]. The extact nature of the thrombin-antithrombin-heparin interaction is not well understood. It was generally believed, that the rate-enhancement is a consequence of heparin binding to antithrombin III [23]. Recent experimental evidence suggests another mechanism, the action of heparin on thrombin rendering it more susceptible to antithrombin III [25—30]. The effect of antithrombin III on the clotting activity of thrombin is more or less known, but there are no data available about the inhibition of the activity of the different thrombin species on small peptide and ester substrates. In the present paper we have examined some properties of the inhibition of the activities of α- and β-thrombin against small synthetic substrates in the presence of antithrombin III and heparin.

We have found that amidase and esterase activities of α- and β-thrombin are inhibited by antithrombin III and, in the case of α-thrombin, the rate of enzyme inactivation is accelerated by heparin.

Materials and Methods

Crude bovine thrombin (Topostasin) was purchased from Hoffman-La Roche, Basel, Switzerland and was further purified by chromatography on a sulphopropyl-Sephadex C-50 column by the method of Lundblad et al. [17]. After separation of α- and β-thrombin, the fractions were gel-filtered on a Sephadex G-25 column (1.1 cm × 20 cm) equilibrated with 25 mM sodium phosphate buffer, pH 7.4.

Antithrombin was purified from normal human plasma by $BaSO_4$ adsorption, Sephadex G-200 gel filtration DEAE-Sephadex A-50 chromatography and isoelectric focusing [31].

The amidase activity of thrombin was determined with benzoyl-L-Phe-Val-Arg-*p*-nitroanilide [32]. The chromogenic substrate was purchased from AB Bofors, Mölndal, Sweden. The 2.5 ml reaction mixture contained 50 μmoles of Tris-imidazole buffer, pH 8.3, 300 μmol of NaCl, 0.25 μmol of substrate and the appropriately diluted thrombin. Incubation was carried out at 37°C. The release of *p*-nitroaniline was determined at 405 nm.

Thrombin esterase activity was assayed spectrophotometrically by the method of Hummel [32]. The reaction mixture contained 200 μmol of sodium phosphate buffer, pH 7.6, 800 μmol of KCl, 4 μmol of substrate (*p*-toluene sulphonylarginine methylester, (TAME), product of Koch-Light Laboratories, England) and thrombin in a final volume of 4.0 ml. The change in differences between the absorbance of the ester and that of the corresponding acid during hydrolysis of the ester was measured at 247 nm.

The inhibition of amidase and esterase activities of the thrombin species in the presence of antithrombin III and heparin were determined by preincubating the thrombin forms with antithrombin III (and heparin) for various times. Thereafter the remaining enzyme activities were measured. Protein concentrations were determined by the method of Hartree [34], using bovine serum albumin as a standard.

Results

α- and β-thrombin were purified from crude bovine preparation and separated by the method of Lundblad et al. [17]. The influence of antithrombin III and heparin on the activities of the two thrombin forms towards small peptide and ester substrates were investigated. Benzoyl-L-Phe-Val-Arg-*p*-nitroanilide (S-2160) was used as peptide substrate, and TAME as ester substrate. The initial rates of hydrolysis are presented in Table I.

The effect of the inhibitor on the amidase activity of α-thrombin is shown on Fig. 1a. Addition of 20 μg antithrombin resulted in 50% inhibition after 3 min preincubation, and 80% of the α-thrombin activity was lost within 6 min. Small amount of heparin (0.002 unit) markedly increased the rate of inactivation.

Antithrombin III seemed to be a less effective inhibitor of β-thrombin amidase activity, as it can be seen on Fig. 1b. During 6 min of preincubation 30 μg of antithrombin gave only 50% inhibition of the enzyme activity. The influence of heparin was much smaller — even if its concentration was higher by an order of magnitude — and did not result in further acceleration of the reaction between β-thrombin and antithrombin III.

The inhibition of the esterase activity of α-thrombin in the presence of antithrombin III was also studied. As is shown in Fig. 2a the inhibition was 'progressive': 3 min preincubation of 10 μg α-thrombin with 50 μg of antithrombin III resulted in 50% inhibition. The reaction showed high sensitivity to heparin. The addition of 0.02 unit of heparin increased the rate of inhibition, i.e. 90% of α-thrombin activity was lost after 1 min preincubation.

Fig. 2b shows the change of esterase activity of β-thrombin in the presence of antithrombin III and heparin. 100 μg inhibitor was necessary to obtain 50% inhibition after 3 min preincubation. The effect of heparin on the inactivation was hardly detectable.

TABLE I

ACTION OF THROMBINS ON AMID AND ESTER SUBSTRATES

Thrombin species	Amidase activity S-2160 * (μmol/min/mg)	Esterase activity TAME ** (μmol/min/mg)
α-Thrombin	13.3	30.2
β-Thrombin	7.7	25.1

* S-2160, benzoyl-L-Phe-Val-Arg-*p*-nitroanilide.

** TAME, *p*-toluene sulphonylarginine methyl ester.

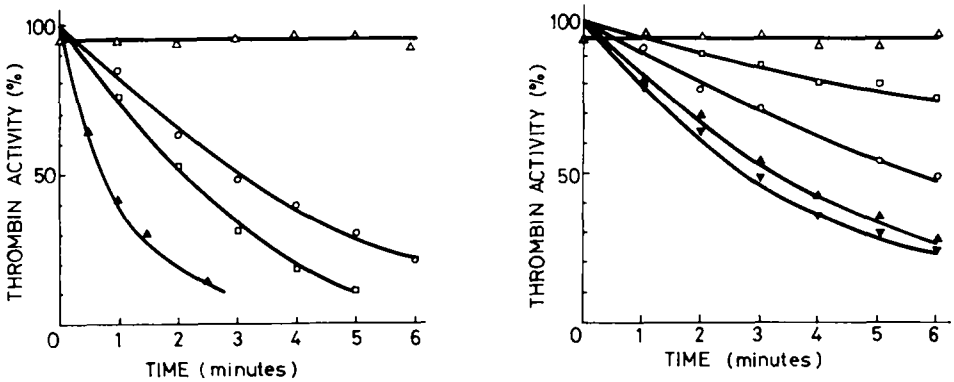

Fig. 1. (a) and (b). Inhibition of the amidase activity of α- and β-thrombin by antithrombin III and heparin. α-Thrombin (Fig. 1a, 1.5 μg of protein) and β-thrombin (Fig. 1b, 2.5 μg of protein) were preincubated at 37°C in 0.2 ml volume in the presence of 6 μmol Tris-imidazole buffer, pH 8.3, and 37 μmol NaCl with the following additives. (a): (△) 0.02 unit heparin; (○) 20 μg antithrombin; (∪) 40 μg antithrombin; (▲) 20 μg antithrombin III plus 0.002 unit heparin. (b): (△) 0.02 unit heparin; (□) 10 μg antithrombin III; (○) 30 μg antithrombin III; (▲) 30 μg antithrombin III plus 0.002 unit heparin; (▼) 30 μg antithrombin III plus 0.02 unit heparin. After incubation for 0—6 min the remaining amidase activities of thrombins were measured.

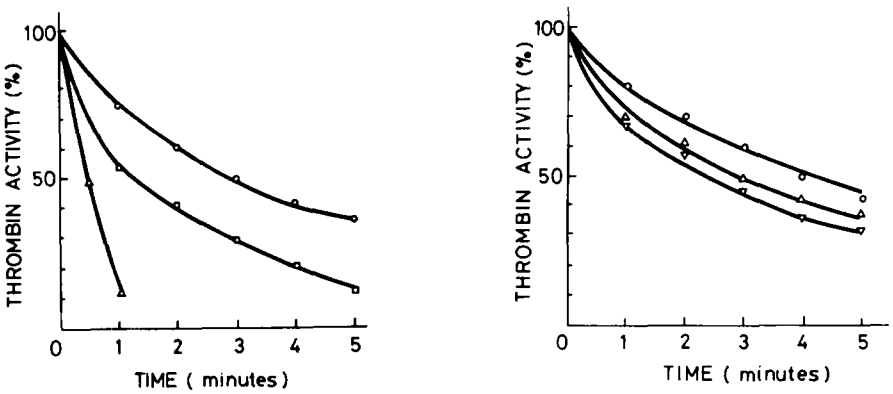

Fig. 2. (a) and (b). Inactivation of the esterase activity of α- and β-thrombin by antithrombin III and heparin. α-Thrombin (a): 10 μg of protein) and β-thrombin (b): 12 μg of protein) were preincubated at 25°C for 0—5 min in 0.5 ml reaction mixture containing 25 μmol sodium phosphate buffer, pH 7.6, and 100 μmol KCl with the following additions. (a): (○) 50 μg antithrombin III; (□) 75 μg antithrombin III and (△) 50 μg antithrombin III plus 0.02 unit heparin. (b): (○) 100 μg antithrombin III; (△) 100 μg antithrombin III plus 0.02 unit heparin; (▽) 100 μg antithrombin III plus 0.2 unit heparin. Thereafter, the remaining esterase activities were determined.

Discussion

In our previous studies we reported, that α- and β-thrombin show different properties concerning their clotting activity, and inactivation by plasma inhibitors, as well as in their heparin sensitivity [20,21]. Great differences were demonstrated only in the clotting activities, i.e. about ten-fold higher specific activity was found for α-thrombin than β-thrombin [20,21]. The amidase activity of β-thrombin (in agreement with the data of Lundblad et al. [19]) is about

50% of that of α-thrombin, whereas the esterase activity is approximately 75—80%.

The enzymic properties of α- and β-thrombin are different for the natural substrate, fibrinogen, although they show similar activities toward synthetic substrates.

Regarding the inhibition of the thrombins activity, after the complex formation with antithrombin III, α-thrombin is no longer capable of acting on either small or large substrate. Similar results were obtained for β-thrombin. The inhibition of the esterase and amidase activities of α- and β-thrombin by antithrombin III is time dependent (progessive) and the amount of the inactivated enzyme is proportional to the concentration of the inhibitor.

Heparin facilitates the complex formation between α-thrombin and antithrombin III. On the other hand, inhibition of the esterase, as well as the amidase activity of β-thrombin in the presence of antithrombin III was only slightly affected by heparin. The difference between the heparin sensitivity of the two species of thrombin seems to provide further evidence in support of the previous suggestions, that heparin facilitates the thrombin-antithrombin reaction by interacting with thrombin [25—30].

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