Inactivation of α - and β -Thrombin by Antithrombin-III, α_2 -Macroglobulin and α_1 -Proteinase Inhibitor

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Inactivation of α - and β -thrombin by α_2 -macroglobulin, by α_1 -proteinase inhibitor and by antithrombin-III and heparin was studied. The amount of α - and β -thrombin inactivated by antithrombin-III was proportional to the concentration of the inhibitor, but the inactivation rates of the two forms of thrombin were different. Heparin facilitated complex-formation between α -thrombin and antithrombin-III, whereas inactivation of β -thrombin by antithrombin was only slightly influenced, even at a heparin concentration two orders of magnitude higher. α_2 -Macroglobulin inhibited both α - and β -thrombin activity similarly, i.e. the amount of α - and β -thrombin inactivated as well as the rates of their inhibition were the same. α_1 -Proteinase inhibitor also formed a complex with α - and β -thrombin, similarly to antithrombin-III, although the inactivation of the enzyme needed high inhibitor concentration and long incubation time. These results suggest that the inactivation of β -thrombin, if it occurs in the plasma, is also controlled by plasma inhibitors.

Thrombin formed in the blood circulation can be inactivated by plasma inhibitors (Abildgaard *et al.*, 1970). Although there is general agreement that antithrombin-III provides the major antithrombin activity of plasma (Abildgaard *et al.*, 1970; Yin *et al.*, 1971; Rosenberg & Damus, 1973) and that α_2 -macroglobulin also contributes to the inhibition of thrombin (Lanchantin *et al.*, 1966; Steinbuch *et al.*, 1967; Harpel, 1973), there are conflicting reports as to the ability of α_1 -proteinase inhibitor to function in this capacity (Borsodi *et al.*, 1975; Learned *et al.*, 1976; Matheson & Travis, 1976).

It has also been found that thrombin exists in multiple active forms (Seegers *et al.*, 1968; Mann & Batt, 1969; Rosenberg & Waugh, 1970; Glover & Shaw, 1971; Lanchantin *et al.*, 1973; Gorman *et al.*, 1976). Three major forms of the enzyme have been observed, i.e. α -, β - and γ -thrombin, which differ substantially in their relative activities toward fibrinogen, although they have identical specific activities toward ester substrates (Mann *et al.*, 1973; Lundblad *et al.*, 1975). It is also known that α -thrombin can be converted into β -thrombin by limited proteolysis (Lundblad *et al.*, 1975). Although the existence of multiple forms of thrombin has been well documented, very little information has been obtained about their biological role.

It has also been found that two forms of thrombin exist with different heparin sensitivity (Machovich *et al.*, 1975a; Machovich, 1975a), and α - and β -thrombin have been proved to be different in their sensitivity to antithrombin-III and heparin (Machovich *et al.*, 1976).

These findings may have great importance from the viewpoint of regulation of blood coagulation and haemostasis; therefore the investigation of the effect of plasma inhibitors on the multiple forms of thrombin is obviously necessary. As it has also been suggested that heparin may affect blood coagulation by its action on thrombin (Machovich, 1975b; Machovich *et al.*, 1975b), the study of the effect of heparin on the inactivation of α - and β -thrombin by antithrombin-III also seems to be reasonable.

The present paper describes some properties and kinetics of the inactivation of α - and β -thrombin by α_2 -macroglobulin and α_1 -proteinase inhibitor, as well as by antithrombin-III and heparin.

Experimental

Materials

Sulphopropyl-Sephadex C-50 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibrinogen (human, grade L) was the product of KABI AB, Stockholm, Sweden.

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Heparin (125 units/mg) dissolved in 0.1M-NaCl was obtained from G. Richter Pharmaceuticals, Budapest, Hungary; 1 unit is defined as 1/130mg of the International Standard Heparin Preparation (Jaques *et al.*, 1973). Other chemicals were purchased from Reanal Fine Chemicals, Budapest, Hungary.

Methods

Crude bovine thrombin (Topostasin) was the product of Hofmann-La Roche, Basel, Switzerland, and it was further purified by chromatography on sulphopropyl-Sephadex C-50 by the method of Lundblad *et al.* (1975). After separation of α - and β -thrombin, the fractions were gel-filtered on a column (1.1 cm $\times$ 20 cm) of Sephadex G-25 equilibrated with 0.05M-sodium phosphate buffer, pH 7.4. The specific activities of α - and β -thrombin were approx. 1800 and 150 NIH units/mg of protein respectively.

Antithrombin-III was purified from human plasma by BaSO₄ adsorption, gel filtration on Sephadex G-200 and chromatography on DEAE-Sephadex A-50, as detailed elsewhere (Borsodi & Bradshaw, 1977). The final product was dissolved or diluted in 0.1M-NaCl. Antithrombin-III was also used that had been purified by the method of Miller-Andersson *et al.* (1974) and supplied by the American Red Cross Fractionation Center, Bethesda, MD, U.S.A.

α_2 -Macroglobulin was purified from human plasma by BaSO₄ adsorption, poly(ethylene glycol) precipitation, DEAE-cellulose chromatography and (NH₄)₂SO₄ precipitation as described by Harpel (1973). A preparation was also used that had been purified by the method of Wickerhauser & Hao (1972) and kindly supplied by the American Red Cross Fractionation Center.

α_1 -Proteinase inhibitor was prepared from the 20% poly(ethylene glycol) supernatant of Cohn fraction IV-1 of human plasma, supplied by the American Red Cross Fractionation Center.

Thrombin activity was assayed in a 0.25 ml reaction mixture containing 5 μ mol of sodium phosphate buffer, pH 7.4, 10 μ mol of NaCl and 500 μ g of fibrinogen. Clotting time was measured in glass tubes with the Hyland Clotek System. Thrombin activity, expressed in NIH units, was calculated from a standard calibration curve established by U.S. Standard Thrombin (human, lot H-1).

The activities of antithrombin-III, α_2 -macroglobulin and α_1 -proteinase inhibitor were assayed in a 0.2 ml reaction mixture containing 5 μ mol of sodium phosphate buffer, pH 7.4, 10 μ mol of NaCl and the appropriately diluted thrombin, thrombin inhibitors and heparin. The reaction mixtures were incubated at 37°C for 0–30 min. After various times 0.05 ml of fibrinogen solution (500 μ g of protein) was added, clotting time measured and the remaining thrombin activity expressed as detailed above.

Protein was determined by the method of Lowry *et al.* (1951) with human serum albumin as a standard.

Concentrations were calculated from mol.wts. of 39000, 28000, 65000, 82000, 54000 and 11000 for α -thrombin (Mann *et al.*, 1973), β -thrombin (Mann *et al.*, 1973), antithrombin-III (Rosenberg & Damus, 1973), α_2 -macroglobulin (Harpel, 1973), α_1 -proteinase inhibitor (Learned *et al.*, 1976) and heparin (Hilborn & Anastassiadis, 1971) respectively.

Results

Inactivation of α - and β -thrombin by antithrombin-III

It has been reported that α - and β -thrombin differ in sensitivity to antithrombin-III and heparin (Machovich *et al.*, 1976); however, the reported experiments were only at one antithrombin-III concentration. Since α - and β -thrombin differed in their molar concentrations at the same clotting activity, the kinetic results obtained may reflect, not only the nature of the two forms of thrombin, but the effect of antithrombin-III concentration as well. Therefore it was necessary to measure the inactivation rates of α - and β -thrombin at various antithrombin-III concentrations, as well as the effect of different heparin concentrations on the α - and β -thrombin inactivation by antithrombin-III.

Fig. 1. Inactivation rate of α -thrombin by antithrombin-III. α -Thrombin (0.9 μ g of protein) in 0.2 ml final volume was incubated at 37°C for 0–8 min in the presence of 5 μ mol of sodium phosphate buffer, pH 7.4, and 10 μ mol of NaCl, with the following additions: ▲, none; Δ, 25 μ g of antithrombin-III; □, 50 μ g of antithrombin-III.

Fig. 2. Inactivation rate of β -thrombin by antithrombin-III. β -Thrombin ($8\mu\text{g}$ of protein) was incubated as detailed in Fig. 1. Additions: $\blacktriangle$, none; $\triangle$, $25\mu\text{g}$ of antithrombin-III; $\square$, $50\mu\text{g}$ of antithrombin-III.

The inactivation kinetics of α -thrombin clotting activity is shown in Fig. 1. The inactivation proceeded with time and depended on the concentration of antithrombin-III, in agreement with the so-called progressive antithrombin activity described in the literature. At $125\mu\text{g}$ of antithrombin-III/ml, about 50% of enzyme activity was lost even after a 2 min incubation.

Fig. 2 shows the inactivation rate of β -thrombin at the same antithrombin-III concentrations as used for α -thrombin inactivation; $125\mu\text{g}$ of antithrombin-III/ml inhibited the enzyme by approx. 40% in a 3 min incubation, whereas twice as much antithrombin-III gave about 60% inactivation during the same period.

The effects of antithrombin-III concentration on α - and β -thrombin activity were also measured. Incubations were carried out for 5 min, since according to the kinetic data (Figs. 1 and 2), during this period and at the chosen concentrations of thrombins and their inhibitor, enzyme activity was inhibited by about 90%. The results in Fig. 3 show that inactivation of both α - and β -thrombin was proportional to the amount of antithrombin-III, although the two forms of thrombin behaved differently. β -Thrombin activity was inhibited by approx. 50% by $125\mu\text{g}$ of antithrombin-III/ml, whereas the same amount of antithrombin-III inactivated α -thrombin by 70%.

As to the effect of heparin, the difference between α - and β -thrombin in sensitivity to heparin was

Fig. 3. Inactivation of α - and β -thrombin by antithrombin-III as a function of inhibitor concentration. α -Thrombin (Δ ; $0.8\mu\text{g}$ of protein) or β -thrombin ($\circ$; $7.5\mu\text{g}$ of protein) in a 0.2ml final volume was incubated at 37°C for 5 min in the presence of $5\mu\text{mol}$ of sodium phosphate buffer, pH 7.4, and $10\mu\text{mol}$ of NaCl.

Fig. 4. Effect of heparin on the inactivation of α - and β -thrombin by antithrombin-III. α -Thrombin (Δ ; $1.1\mu\text{g}$ of protein) or β -thrombin ($\circ$; $7.9\mu\text{g}$ of protein) in a 0.2ml final volume was incubated at 37°C for 1 min in the presence of $5\mu\text{mol}$ of sodium phosphate buffer, pH 7.4, $10\mu\text{mol}$ of NaCl and various concentrations of heparin.

much more pronounced than the difference against the inactivation effect of antithrombin-III. The same activity of α - and β -thrombin was incubated for 1 min in the presence of a constant amount of antithrombin-III and various concentrations of heparin (Fig. 4). The concentration of antithrombin-III used for this experiment ($250\mu\text{g}/\text{ml}$) was able to inactivate

Fig. 5. Inhibition of the activity of α - and β -thrombin by α_2 -macroglobulin

α -Thrombin (Δ ; 1 μ g of protein) or β -thrombin ($\circ$; 8 μ g of protein) in a 0.2 ml final volume was incubated at 37°C for 30 min in the presence of 5 μ mol of sodium phosphate buffer, pH 7.4, 10 μ mol of NaCl and various concentrations of α_2 -macroglobulin.

more than 90% of α - and β -thrombin during 5 and 8 min of incubation respectively. Antithrombin-III at this concentration was in excess for both thrombins. Therefore the heparin effect under this condition reflects the character of α - as well as β -thrombin. Without heparin, more α -thrombin was inactivated by antithrombin-III than β -thrombin, in accordance with the results demonstrated in Fig. 3. In the presence of heparin, however, α - and β -thrombin behaved extremely differently: α -thrombin was considerably inactivated at a low heparin concentration of 0.0025 unit/ml (thrombin activity was not measurable in our assay system at this heparin concentration), whereas β -thrombin inactivation by antithrombin-III changed only slightly even at a heparin concentration two orders of magnitude higher. The decrease in the activity of β -thrombin in the presence of 0.005 unit of heparin/ml was scarcely different from that at 0.2 unit of heparin/ml.

Inhibition of the activity of α - and β -thrombin by α_2 -macroglobulin

α - and β -Thrombin were incubated at 37°C for 30 min in the presence of different concentrations of α_2 -macroglobulin. The inhibition of both kinds of thrombin activity increased with the concentrations of α_2 -macroglobulin (Fig. 5). Furthermore enzyme inhibition by α_2 -macroglobulin was similar for α - and β -thrombin, i.e. 50% of enzyme activity was inhibited by the same amount of α_2 -macroglobulin.

Fig. 6. Inactivation of α - and β -thrombin by α_1 -proteinase inhibitor as a function of the inhibitor concentration α -Thrombin (Δ ; 1.2 μ g of protein) or β -thrombin ($\circ$; 9.5 μ g of protein) in a 0.2 ml final volume was incubated at 37°C for 30 min in the presence of 5 μ mol of sodium phosphate buffer, pH 7.4, 10 μ mol of NaCl and various concentrations of α_1 -proteinase inhibitor.

Fig. 7. Inactivation rate of α -thrombin by α_1 -proteinase inhibitor

α -Thrombin (1 μ g of protein) in 0.2 ml final volume was incubated at 37°C for 0–30 min in the presence of 5 μ mol of sodium phosphate buffer, pH 7.4, and 10 μ mol of NaCl with the following additions: $\blacktriangle$, none; Δ , 50 μ g of α_1 -proteinase inhibitor; $\square$, 100 μ g of α_1 -proteinase inhibitor.

Inactivation of α - and β -thrombin by α_1 -proteinase inhibitor

Matheson & Travis (1976) showed that when a short (5 min) time of incubation of thrombin and α_1 -proteinase inhibitor was used, thrombin was not significantly inhibited by α_1 -proteinase inhibitor. Therefore in our present experiments α - and β -thrombin were incubated for 30 min at 37°C in the

Fig. 8. Inactivation rate of β -thrombin by α_1 -proteinase inhibitor

β -Thrombin (8 μ g of protein) was incubated as detailed in Fig. 7. Additions: ▲, none; □, 100 μ g of α_1 -proteinase inhibitor; ■, 200 μ g of α_1 -proteinase inhibitor.

presence of different concentrations of α_1 -proteinase inhibitor. Under these conditions, α_1 -proteinase inhibitor inactivated α - and β -thrombin, although α -thrombin was more strongly inhibited than was β -thrombin; 50% of α -thrombin was inactivated already at a concentration of 300 μ g of inhibitor/ml, whereas the same percentage inhibition of β -thrombin activity was obtained at about 600 μ g of α_1 -proteinase inhibitor/ml (Fig. 6).

On the basis of these data, it seemed worth while to examine the kinetic difference between α - and β -thrombin inactivation by α_1 -proteinase inhibitor. As shown in Fig. 7, the rate of α -thrombin inactivation increased with increasing concentration of the inhibitor. In the presence of 500 μ g of α_1 -proteinase inhibitor/ml, 90% of enzyme activity was lost in a 30 min incubation when α -thrombin concentration was 5 μ g/ml. Similar results were obtained with β -thrombin, except that for the same percentage inactivation twice as much α_1 -proteinase inhibitor was necessary (Fig. 8).

Discussion

There is a general agreement that antithrombin-III and α_2 -macroglobulin provide the major antithrombin activity of plasma. However, the finding of Matheson & Travis (1976) raises the possibility that α_1 -proteinase inhibitor contributes to the function of antithrombin-III and α_2 -macroglobulin. Their data show that α_1 -proteinase inhibitor can be an inhibitor of thrombin *in vitro* at relatively high concentrations or during a long incubation period. Our results agree with this conclusion. As the concentration of α_1 -proteinase inhibitor is probably 10–20-fold

higher than the maximum possible concentration of thrombin or antithrombin-III *in vivo* (Heimburger *et al.*, 1971), the relatively high content of this inhibitor may indeed contribute to the enzyme inactivation, especially under circumstances when other plasma inhibitor concentrations decreased. This problem, however, is more complicated, since there is no information about the effect of the fibrinogen substrate on the reaction of thrombin with its inhibitors, especially when thrombin forms in the blood circulation.

Nevertheless, our experiments show that a concentration of approx. 10–20 μ M- α_1 -proteinase inhibitor can inhibit more than 90% of 0.13 μ M- α -thrombin or 1.4 μ M- β -thrombin. Antithrombin-III, however, is a more potent inhibitor of thrombin, particularly in the presence of heparin. Even 2 μ M-antithrombin-III is able to inactivate the same amount of enzyme in 5 min as a 10-fold higher concentration of α_1 -proteinase inhibitor during 30 min. When heparin is also present, even at a concentration as low as 2 nM, the inactivation rate increases, and about 0.1 μ M-thrombin is inactivated by antithrombin-III in 1 min or even less. This latter result suggests that the effective heparin concentration for α -thrombin is not equimolar with either the enzyme or with its inhibitor. This conclusion is in agreement with the findings of Björk & Nordenman (1976).

α_2 -Macroglobulin is also able to inhibit thrombin activity, i.e. 6 μ M- α_2 -macroglobulin inactivates more than 90% of 0.13 μ M- α -thrombin as well as 1.4 μ M- β -thrombin in 30 min, and the inactivation rates are the same for both thrombins.

In spite of the fact that different amounts of antithrombin-III and α_1 -proteinase inhibitor are required for the inactivation of the thrombins, both distinguish between α - and β -thrombin in the rate at which they inactivate. On the other hand, α_2 -macroglobulin shows the same inactivation rate for both thrombins.

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