Inactivation of Human Thrombin in the Presence of Human α_1 -Proteinase Inhibitor

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Both the clotting and esterase activities of thrombin are inhibited by α_1 -proteinase inhibitor (α_1 -antitrypsin). The inhibition is a time- and temperature-dependent reaction which is proportional to the molar ratio of thrombin to inhibitor. Both the active-site serine residue of thrombin and the reactive-site lysine residue of α_1 -proteinase inhibitor are involved. α_1 -Proteinase inhibitor forms a 1:1 complex with thrombin that is comparable with the complex formed with trypsin and other proteinases. Incubation of the inhibitor with excess of thrombin, however, results in inactivation of nearly all the enzyme, even though only as much complex is formed as α_1 -proteinase inhibitor present. A portion of the remaining thrombin apparently aggregates. These results suggest that the mechanism for inhibition of thrombin may not be exactly the same as for trypsin, which is inhibited only to the extent to which complex is formed.

Antithrombin III and α_2 -macroglobulin have been considered by some investigators to be the major inhibitors of thrombin in human plasma (Heimburger *et al.*, 1971; Rosenberg & Damus, 1973). α_1 -Proteinase inhibitor, formerly called α_1 -antitrypsin, which inhibits many other proteinases (Heimburger *et al.*, 1971; Learned *et al.*, 1976) was not considered to be an inhibitor of thrombin by these investigators. Workers in some laboratories, however, found that thrombin was inhibited by α_1 -proteinase inhibitor, by using both the conventional clotting assay (Rimon *et al.*, 1966) and an immunological assay (Lane *et al.*, 1975). By the latter method, Lane *et al.* (1975) found that approx. 50% of human plasma antithrombin activity was due to antithrombin III, and 25% each to α_2 -macroglobulin and α_1 -proteinase inhibitor. Although the main thrombin inhibitor, antithrombin III, very rapidly inhibits thrombin in the presence of heparin, its concentration in plasma is probably only about two to four times higher than the maximum possible thrombin concentration (Heimburger *et al.*, 1971). The concentration of α_1 -proteinase inhibitor is probably about 10-20 times higher than the concentration of thrombin. Thus if this protein does inhibit thrombin, it might be expected to have some significance in the regulation of thrombin activity *in vivo*, especially in antithrombin III deficiency. None of these studies, however, have been carried out with highly purified preparations of both α_1 -proteinase inhibitor and thrombin. The presence of small amounts of contaminating inhibitors, proteinases, or other factors in partially purified preparations might lead to erroneous conclusions

about the inhibition of thrombin by α_1 -proteinase inhibitor.

The present study was undertaken in an effort to resolve the previous conflicting results on the inhibition of thrombin by α_1 -proteinase inhibitor. By using highly purified proteins we demonstrate that α_1 -proteinase inhibitor does inhibit thrombin in a time- and temperature-dependent reaction. In addition, data are presented which indicate that the inhibition, although probably involving formation of a stoichiometric complex, perhaps also involves interactions not observed during the inhibition of other proteinases by α_1 -proteinase inhibitor.

Experimental

Materials

Outdated human plasma was obtained from both St. Mary's Hospital and Athens General Hospital, Athens, GA, U.S.A. N.I.H. standard thrombin (lot B-3) was obtained from Dr. David L. Aronson, Division of Biological Standards, National Institutes of Health, Bethesda, MD, U.S.A. Human serum, used in prothrombin activation, was prepared immediately before use. Fresh blood was drawn into a Vacutainer clot tube (purchased from Becton-Dickinson and Co., Rutherford, NJ, U.S.A.), allowed to clot for 1 h, and then the clot removed by centrifugation at 3000g for 15 min. Thromboplastin was prepared from rabbit brains as described by Magnusson (1970). Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Sulphopropyl-Sephadex C-50, di-isopropyl phosphorofluoridate, fibrinogen (F-4000, fraction 1,

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type 1), bovine serum albumin, tosyl-L-arginine methyl ester (Tos-Arg-OMe) and *N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Polyethylene glycol 6000 was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Human α_1 -proteinase inhibitor was prepared by the method of Pannell *et al.* (1974). Pig trypsin was prepared by the method of Travis & Liener (1965).

Methods

Measurement of protein concentration. Protein concentrations were determined by absorbance measurements at 280 nm. The $E_{280}^{1\%}$ was assumed to be 19.5 for thrombin (Winzor & Scheraga, 1964) and 5.3 for α_1 -proteinase inhibitor (Pannell *et al.*, 1974). Protein concentrations of thrombin were also measured by the method of Lowry *et al.* (1951) and found to be virtually identical with those measured spectrophotometrically with bovine serum albumin as a standard.

Modification of lysine residues. Lysine residues in α_1 -proteinase inhibitor were modified with 2,3-dimethylmaleic anhydride essentially by the method of Butler *et al.* (1969) as modified by Gertler (1971) and with citraconic anhydride as described by Atassi & Habeeb (1972). α_1 -Proteinase inhibitor, in 0.05 M-Tris/HCl (pH 8.8)/0.05 M-NaCl, was incubated for 45 min with citraconic anhydride (89 μ mol/mg of protein) or for 4 h with 2,3-dimethylmaleic anhydride (12 μ mol/mg of protein). After incubation, each reaction mixture was dialysed overnight, with two changes, against 4 litres of 0.05 M-Tris/HCl (pH 8.8)/0.05 M-NaCl at 4°C. To reverse the modification, the citraconic anhydride-treated protein was dialysed for 2 days against 4 litres of 0.05 M-sodium phosphate buffer, pH 6.0, and the 2,3-dimethylmaleic anhydride-treated protein was dialysed for 24 h against 4 litres of 0.05 M-sodium phosphate buffer, pH 6.7.

Sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis. The procedure used was a modification of that of Laemmli (1970) as described by Baugh & Travis (1976), by using 9% (w/v) polyacrylamide-gel slabs (112 mm $\times$ 77 mm). The samples were prepared by extensive dialysis against 0.02 M-sodium phosphate buffer, pH 7.0, and concentration in a Buchler Instruments Rotary Evapomix at 34°C. Each sample, containing a final concentration of 1% (w/v) sodium dodecyl sulphate, was then heated at 100°C for 5 min. After cooling, 0.05% Bromophenol Blue in 50% (v/v) glycerol was added. The sample volumes applied were 50–70 μ l, containing approx. 20 μ g of each protein. The gels were fixed in 20% (w/v) sulphosalicylic acid for 60 min. The sodium dodecyl sulphate was removed from the gels by soaking overnight in 7.5% (v/v) acetic acid with three changes. The protein was stained with 0.25% Coomassie Brilliant Blue R in methanol/water/acetic

acid (5:5:1, by vol.) at 60°C for 30 min, then destained in 7.5% acetic acid.

Sephadex G-75 chromatography. The components of incubation mixtures of thrombin and α_1 -proteinase inhibitor were separated on a column (1.2 cm $\times$ 116.8 cm) of Sephadex G-75. The column was equilibrated with 0.1 M-sodium phosphate buffer (pH 7.0)/0.15 M-NaCl; fractions (1 ml) were collected and the E_{280} was determined.

Assays. Thrombin clotting activity was assayed by the method of Lundblad (1971) with the following modifications: thrombin was diluted in 0.1 M-Tris/HCl, pH 7.3, and the clotting reaction mixture had a final polyethylene glycol concentration of 0.66% (w/v) (Fasco & Fenton, 1973). Clotting times were converted into thrombin N.I.H. units by comparison with a standard curve prepared from N.I.H. standard thrombin (lot B-3) (containing 21.7 N.I.H. units/mg), under the same conditions. A thrombin N.I.H. unit is defined as that amount of thrombin which will clot 1 ml of a standardized fibrinogen solution in 15 ± 0.5 s. Thrombin clotting activity measured by this assay was within 10% of that measured by a modification of the standard N.I.H. procedure (Mann *et al.*, 1971). Thrombin esterase activity was assayed spectrophotometrically at 247 nm by using Tos-Arg-OMe (Hummel, 1959).

The inhibitory activity of α_1 -proteinase inhibitor against trypsin was checked periodically to ensure that fully active (sp. activity 18–20 units/ E_{280} , where 1 unit of activity is defined as a ΔE_{253} of 1.0/min) α_1 -proteinase inhibitor would be used in the experiments with thrombin. Assays were performed spectrophotometrically at 253 nm and 25°C with ox or pig trypsin and Bz-Arg-OEt in 0.05 M-Tris/HCl, pH 8.0, as described by Pannell *et al.* (1974).

The inhibition of thrombin by α_1 -proteinase inhibitor was assayed by measuring the clotting activity or esterolytic activity of thrombin after incubation with the inhibitor. These activities were compared with the control activity of thrombin alone. Reaction mixtures were incubated at 22°, 30° or 37°C, and contained 0.05 M-Tris/HCl, pH 7.3, or 0.1 M-sodium phosphate buffer, pH 7.0, 0.15 M-NaCl, and appropriate amounts of thrombin and α_1 -proteinase inhibitor in a final volume of 0.5 ml. Samples were removed at various times, diluted appropriately, and assayed.

Isolation of human thrombin. Human prothrombin was partially purified up to and including the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step by the method of Bajaj & Mann (1973). The 40–60%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate from 500 ml of plasma was then dissolved in 0.025 M-sodium citrate buffer, pH 6.0, and made 0.31 M with di-isopropyl phosphorofluoridate in propan-2-ol. The di-isopropyl phosphorofluoridate-treated protein was dialysed for 1 h against 2 litres of 0.025 M-sodium citrate buffer, pH 6.0, and then against

2 litres (each time) of 0.1 M-Tris/HCl, pH 7.3, overnight with three changes. The prothrombin was activated to thrombin by incubation at 37°C for 90 min with 30 ml of the diffusate, 1.15 ml of freshly prepared human serum, and 3.70 ml of rabbit brain thromboplastin in 0.1 M-Tris/HCl (pH 7.3)/14 mM-NaCl/3.2 mM-CaCl₂. The activation mixture was dialysed for 2 h with two changes against 2 litres of 0.025 M-sodium phosphate buffer, pH 6.5, and applied to a column (2.4 cm $\times$ 10.0 cm) of sulphopropyl-Sephadex C-50 equilibrated with the same buffer. The column was washed with the equilibrating buffer and 0.1 M-sodium phosphate buffer, pH 6.5, and the thrombin eluted with 0.25 M-sodium phosphate buffer, pH 6.5, as described by Lundblad (1971).

Specific activities of 3500 N.I.H. units/mg were obtained as a routine. Occasionally much higher specific activities were obtained, up to 9260 N.I.H. units/mg. We have not attempted to determine the reason for such high specific activities. However, the age of the serum used in the activation of prothrombin appears to be responsible, at least in part, for the specific activities obtained. If the serum is prepared just before use, specific activities are at least 3500 N.I.H. units/mg and usually higher. If it is a day or more older, the specific activities are usually 2200 N.I.H. units/mg and lower but occasionally higher. Serum is used as a source of factor V, which is not very stable. Factor V is usually partially purified before use (Rosenberg & Damus, 1973) or purchased commercially (Bajaj & Mann, 1973). Thus use of fresh serum as a source of factor V may be the reason for high specific activities. It is noteworthy that whether the specific activity of the thrombin is 2000 or 8000 units/mg, only one band is seen on a sodium dodecyl sulphate/polyacrylamide-gel slab and one peak on a Sephadex G-75 column chromatography. In all cases, the thrombin obtained is extremely stable. There is no loss of clotting or esterolytic activity even after 64 h at 37°C, or after 3 months at 4°C in the presence of NaN₃.

Results

Inhibition of thrombin activity

The demonstration of the inhibition of thrombin clotting activity by α_1 -proteinase inhibitor is shown in Fig. 1. The inhibition proceeds with time and is dependent on the relative molar ratios of thrombin to α_1 -proteinase inhibitor. The initial rate of inhibition increases from 4.9 pmol of thrombin inhibited/min per ml at a molar ratio of 1:1, to 11.2 at 1:2, 22.2 at 1:5, and 37.0 at 1:10. Thrombin incubated alone loses no activity. In all cases the initial concentration of thrombin was 0.68 μ M. If the α_1 -proteinase inhibitor had previously been heated to 60°C for 10 min, however, it no longer had any effect on thrombin clotting activity or trypsin esterolytic activity.

Fig. 1. *Inhibition of thrombin clotting activity and esterolytic activity by α_1 -proteinase inhibitor as a function of time and molar ratio*

For inhibition of clotting activity, thrombin (0.68 μ M) was incubated with α_1 -proteinase inhibitor (0, 0.68, 1.36, 3.38, or 6.75 μ M) in 0.05 M-Tris/HCl (pH 7.3)/0.15 M-NaCl or 0.1 M-sodium phosphate buffer, pH 7.0, at 37°C. At the time-intervals indicated, samples were removed and the clotting activity assayed as described under 'Methods'. For inhibition of esterolytic activity, thrombin (1.08 μ M) was incubated with α_1 -proteinase inhibitor (5.04 μ M) in 0.05 M-Tris/HCl (pH 7.3)/0.15 M-NaCl. At the time-intervals indicated, samples were removed and assayed spectrophotometrically by using Tos-Arg-OMe as a substrate, as described under 'Methods'. (●) thrombin alone; (○) thrombin and α_1 -proteinase inhibitor, molar ratio 1:1; (▲) thrombin and α_1 -proteinase inhibitor, 1:2; (△) Thrombin and α_1 -proteinase inhibitor, 1:5; (■) thrombin and α_1 -proteinase inhibitor, 1:10; (□) esterolytic assay of thrombin and α_1 -proteinase inhibitor, 1:5.

The esterolytic activity of thrombin is also inhibited by α_1 -proteinase inhibitor, as shown in Fig. 1. The initial rate of inhibition of thrombin esterolytic activity is 30.8 pmol/min per ml at a thrombin/(α_1 -proteinase inhibitor) molar ratio of 1:5. The initial concentration of thrombin in the esterolytic assay was 1.08 μ M.

The α_1 -proteinase-inhibitor preparations used were found to be free of any detectable amounts of contaminating proteinases or antithrombin III. Treatment of α_1 -proteinase-inhibitor preparations with di-isopropyl phosphorofluoridate, an irreversible

inhibitor of serine proteinases, did not affect the rate of inhibition of thrombin by α_1 -proteinase inhibitor. Also α_1 -proteinase-inhibitor preparations did not exhibit any reaction with azocasein, a proteinase substrate, even after incubation for 54h, compared with controls incubated for the same lengths of time. In addition, α_1 -proteinase inhibitor was shown to be free of antithrombin III by the Ouchterlony (1949) double-diffusion technique against anti-(antithrombin III). Diluted standard human plasma against anti-(antithrombin III) or anti-(α_1 -proteinase inhibitor) showed that antithrombin III is detectable down to $0.65 \mu\text{g}/5 \mu\text{l}$ and α_1 -proteinase inhibitor to $0.52 \mu\text{g}/5 \mu\text{l}$. Since antithrombin III acts as an inhibitor in a stoichiometric manner, the possible presence of antithrombin III in α_1 -proteinase-inhibitor preparations in concentrations lower than this could not account for the inhibition of thrombin observed. Also, the addition of heparin, which stimulates the rate of thrombin inhibition by antithrombin III, had no effect on the rate of inhibition of thrombin by the α_1 -proteinase inhibitor preparations used.

Protection by substrate

The presence of the esterolytic substrate, Tos-Arg-OMe, in the reaction mixture for clotting assays protected thrombin clotting activity from inhibition by α_1 -proteinase inhibitor. These results are shown in Fig. 2. In the presence of 0.5mM-Tos-Arg-OMe, the rate of inhibition is 59% of the rate in its absence; with 1.0mM, the rate is 41% of normal; and with 2.0mM, the rate is 22% of normal. Tos-Arg-OMe alone, at these concentrations, has no effect on the clotting activity. Competition of the small synthetic substrate, Tos-Arg-OMe, with the inhibitor, α_1 -proteinase inhibitor, suggests that the latter is probably acting at the active site of thrombin.

Effect of modifying α_1 -proteinase inhibitor

To establish whether the reactive centre of α_1 -proteinase inhibitor is also involved, acylation of α_1 -proteinase inhibitor was performed. Lysine has been shown to be the reactive centre of α_1 -proteinase

Fig. 2. Protection of thrombin clotting activity from α_1 -proteinase inhibitor by an ester substrate, Tos-Arg-OMe

Thrombin ($0.68 \mu\text{M}$) was incubated with α_1 -proteinase inhibitor ($3.38 \mu\text{M}$) in 0.05M-Tris/HCl ($\text{pH} 7.3$)/ 0.15M-NaCl in the absence and presence of Tos-Arg-OMe. At the time-intervals indicated, samples were removed and the clotting activity was assayed as described under 'Methods'. (●) Thrombin and 2.0mM-Tos-Arg-OMe; (○) thrombin and α_1 -proteinase inhibitor, 1:5, and no Tos-Arg-OMe; (▲) thrombin and α_1 -proteinase inhibitor, 1:5, and 0.5mM-Tos-Arg-OMe; (△) thrombin and α_1 -proteinase inhibitor, 1:5, and 1.0mM-Tos-Arg-OMe; (■) thrombin and α_1 -proteinase inhibitor, 1:5, and 2.0mM-Tos-Arg-OMe.

inhibitor towards other proteinases (Heimburger *et al.*, 1971). Modification of lysine residues in native α_1 -proteinase inhibitor by citraconic anhydride or 2,3-dimethylmaleic anhydride in alkaline solution resulted in the loss of inhibitory activity of α_1 -proteinase inhibitor toward both trypsin and thrombin. By dialysis of α_1 -proteinase inhibitor, modified by either 2,3-dimethylmaleic anhydride or citraconic anhydride, against slightly acidic solutions to reverse the modification, the inhibitory activity was partially restored to approx. 30% of the original inhibitory activity. This is a rather low recovery, but the protein becomes denatured at lower pH where recovery might be expected to be more nearly complete.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

In all other cases in which α_1 -proteinase inhibitor inhibits a proteinase, the inhibition is caused by the irreversible binding of the reactive centre of α_1 -proteinase to the active site of the proteinase (Travis

et al., 1974; Baugh & Travis, 1976). Indeed, in the inhibition of thrombin, a complex also is formed between this proteinase and α_1 -proteinase inhibitor, as shown by sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis of incubation mixtures of the two proteins at different molar ratios (Plate 1). Exactly the same pattern also occurs in the presence of 2-mercaptoethanol. From a plot of molecular weight against $\log(\text{relative mobility})$, comparison with standards indicates that the mol.wt. of the complex is approx. 90000. A 1:1 complex of α_1 -proteinase inhibitor and thrombin would be expected to have a mol.wt. of approx. 87000. Even at a thrombin/ (α_1 -proteinase inhibitor) molar ratio of 1:4, however, some thrombin remains uncomplexed.

Sephadex G-75 chromatography

A complex of α_1 -proteinase inhibitor and thrombin can also be demonstrated by Sephadex G-75 chromatography of an incubation mixture of α_1 -proteinase inhibitor and thrombin (Fig. 3, curve A, peak I).

Fig. 3. Gel-filtration pattern for an incubation mixture of thrombin and α_1 -proteinase inhibitor

Thrombin (15.6 nmol) and α_1 -proteinase inhibitor (18.2 nmol) in 0.1 M-sodium phosphate buffer (pH 7.0)/0.15 M-NaCl were mixed and incubated at 37°C for 16 h until the thrombin clotting activity remaining was 7% of the original activity. Thrombin (15.6 nmol) was also incubated at 37°C alone for 16 h with no loss of activity. Each incubation solution was chromatographed on a column (1.2 cm $\times$ 116.8 cm) of Sephadex G-75 equilibrated with 0.1 M-sodium phosphate buffer (pH 7.0)/0.15 M-NaCl. The recovery of protein was 92% for the thrombin- α_1 -proteinase inhibitor mixture and 75% for thrombin alone. Curve A (●), thrombin and α_1 -proteinase inhibitor; curve B (○), thrombin.

This peak contains no α_1 -proteinase inhibitor or thrombin activity. Elution volumes of controls indicate that peak II contains uncomplexed α_1 -proteinase inhibitor, and peak III contains uncomplexed thrombin. The initial concentrations were 15.6 nmol of thrombin and 18.2 nmol of α_1 -proteinase inhibitor, an approximately 1:1 molar ratio. Peak III, however, contains 28% of the original amount of thrombin applied, or 4.2 nmol. This amount of thrombin before incubation with α_1 -proteinase inhibitor would be expected to contain 1170 N.I.H. units, but only 90 N.I.H. units can be detected. This is only 8% of the activity that should be present in uncomplexed thrombin if complex-formation is required for inhibition. One possible explanation is that there is some inactive thrombin present in the original thrombin preparation. However, the amount present in the thrombin peak appears to be approx. 30%, even though the specific activity of various preparations has ranged from 4200 to 9260 N.I.H. units/mg. Curve B represents the same original amount of thrombin as in curve A, treated in the same way but not exposed to α_1 -proteinase inhibitor. Recovery of protein in the peak was 75%, and all of the original clotting activity corresponding to that amount of protein was present, indicating that thrombin activity is not lost merely by passage through the column. When thrombin was treated first with di-isopropyl phosphorofluoridate, then incubated as usual with α_1 -proteinase inhibitor, and passed through a Sephadex G-75 column, no complex was formed, and the inhibitor and thrombin appeared at their usual positions, indicating again that the active site of thrombin is involved in its inhibition by α_1 -proteinase inhibitor.

Effect of temperature

Changes in temperature over about 15°C would not be expected to have any detectable effect on the rate and extent of rapid irreversible complex-formation by the relatively slow detection methods used here. However, when the molar ratio of thrombin to α_1 -proteinase inhibitor is kept constant at 1:5 and the temperature of incubation is varied, there is a great difference in the rate of inhibition of thrombin by α_1 -proteinase inhibitor, as shown in Fig. 4. The rate varies from 22.2 pmol of thrombin inhibited/min per ml at 37°C to 8.4 at 30°C, to 3.7 pmol/min per ml at 22°C. Thus the rate at 37°C is nearly three times that at 30°C and six times that at 22°C.

Effect of excess of thrombin

Assuming a ratio of 1:1 for thrombin/(α_1 -proteinase inhibitor) in the complex, a molar ratio of thrombin to α_1 -proteinase inhibitor of 10:1 would be expected to yield a total inhibition of 10%, and a molar ratio of 5:1 should yield a 20% total inhibition.

However, the extent of inhibition under these conditions is much greater than this, as shown in Fig. 4. At a thrombin/(α_1 -proteinase inhibitor) molar ratio of 5:1, the inhibition is 56% in 4 h, 73% in 9 h and 94% in 24 h. At the 10:1 molar ratio, the inhibition is 53% in 3 h, 62% in 5 h and 85% in 22 h. At even the longest times of incubation, thrombin alone retains its full activity. Although a long incubation is required under these conditions, nearly complete inhibition of thrombin is obtained.

Also, these last results show that the rate of inhibition of thrombin depends not only on the molar ratios but also on the absolute concentrations of thrombin and α_1 -proteinase inhibitor. The rate of inhibition is actually faster at a 10:1 than at a 5:1 molar ratio of thrombin to α_1 -proteinase inhibitor, since the concentrations of thrombin and α_1 -proteinase inhibitor were much higher in the former case. The initial concentration of thrombin at the 10:1 molar ratio was

Fig. 4. Inhibition of thrombin clotting activity by α_1 -proteinase inhibitor as a function of time at various temperatures and molar ratios

Thrombin (0.68 μ M) was incubated with α_1 -proteinase inhibitor (3.38 μ M) at 22°C, 30°C, and 37°C and assayed at the time-intervals indicated as described in Fig. 1. Also, thrombin (1.32 μ M) and α_1 -proteinase inhibitor (0.27 μ M), for a 5:1 molar ratio, and thrombin (10.79 μ M) and α_1 -proteinase inhibitor (1.04 μ M), for a 10:1 molar ratio, were incubated at 37°C and assayed at the time-intervals indicated as described in Fig. 1. (●) Thrombin alone at 22°C, 30°C, or 37°C; (○) thrombin and α_1 -proteinase inhibitor, 1:5, at 22°C; (■) thrombin and α_1 -proteinase inhibitor, 1:5, at 30°C; (□) thrombin and α_1 -proteinase inhibitor, 1:5, at 22°C; (▲) thrombin and α_1 -proteinase inhibitor, 5:1, at 37°C; (△) thrombin and α_1 -proteinase inhibitor, 10:1, at 37°C.

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis of thrombin, α₁-proteinase inhibitor and thrombin-α₁-proteinase inhibitor complex formed at various molar ratios

Electrophoresis was at pH 6.8, in a 9% (w/v) gel (1) thrombin (Thr) (38 μg); (2) 1:1 molar ratio, thrombin (25 μg)/α₁-proteinase inhibitor (α₁-PI) (37 μg); (3) 1:2 molar ratio, thrombin (18 μg)/α₁-proteinase inhibitor (55 μg); (4) 1:4 molar ratio, thrombin (12 μg)/α₁-proteinase inhibitor (72 μg); (5) α₁-proteinase inhibitor (26 μg); (6) mol.wt. markers: bovine serum albumin (BSA) (66200) (15 μg), ovalbumin (OA) (43000) (15 μg), trypsinogen (Tg) (23500) (15 μg).

Fig. 5. Sephadex G-75 chromatography for incubation mixtures of α_1 -proteinase inhibitor and excess of thrombin

Thrombin (15.8 nmol) and α_1 -proteinase inhibitor (a, 6.6 nmol; b, 3.2 nmol) were incubated together and chromatographed on Sephadex G-75 as described in Fig. 3.

10.7 μM , eight times the concentration at the 5:1 molar ratio, 1.32 μM . The rates of inhibition were 31.4 and 3.3 pmol of thrombin inhibited/min per ml respectively, a ninefold difference. In fact, if concentrations of thrombin and α_1 -proteinase inhibitor are chosen to represent the estimated concentrations *in vivo*, a much more rapid rate of inhibition occurs (at least tenfold more rapid) than in all the experiments discussed above.

When an incubation mixture of thrombin and α_1 -proteinase inhibitor containing excess of thrombin is chromatographed on a column of Sephadex G-75, the pattern is somewhat different from that of a 1:1 mixture. The result of chromatography of a 2:1 thrombin/(α_1 -proteinase inhibitor) molar ratio is shown in Fig. 5. Only 10% of the original thrombin activity remained after incubation with α_1 -proteinase inhibitor. The concentration of thrombin was 3.77 μM with a total of 17.0 nmol. On the basis of the E_{280} in the peaks and the total amount (nmol) and E_{280} of thrombin at the start, 9.55 nmol or 55% of the thrombin was recovered in the thrombin peak (peak II) and 7.50 nmol of thrombin and all of the α_1 -proteinase inhibitor (6.34 nmol) in the thrombin-inhibitor complex peak (peak I). The result of chromatography of a 5:1 thrombin/(α_1 -proteinase inhibitor) molar ratio is shown in Fig. 5(b). The original concentration of thrombin was the same as in Fig. 5(a), with a total of 22.4 nmol. The thrombin-inhibitor complex peak (peak I) contains 2.82 nmol of thrombin and 3.15 nmol of α_1 -proteinase inhibitor, approximately the amount

of complex expected from a 5:1 molar ratio. In the thrombin peak (peak III), 12.5 nmol was present, about 55% of the total. However, in this case, another peak (peak II) appeared at the approximate position of α_1 -proteinase inhibitor. A trace of clotting activity could be detected in this peak. No α_1 -proteinase inhibitor could be detected by the Ouchterlony immunodiffusion technique against anti-(α_1 -proteinase inhibitor), though a solution of known α_1 -proteinase inhibitor of similar protein concentration gave a very strong precipitin line. Also, all of the α_1 -proteinase inhibitor added to the original incubation mixture was in the complex. In other similar experiments, peak II was not seen in the intermediate position but a peak was found at the void volume. Since thrombin alone does not aggregate at the protein concentrations used in these experiments, it appears that aggregation of thrombin may be a result of exposure to α_1 -proteinase inhibitor when the concentration of α_1 -proteinase inhibitor is too low to form a complex with all of the thrombin.

Discussion

The question of whether thrombin is inactivated by α_1 -proteinase inhibitor has been under debate for several years. Some studies have indicated that α_1 -proteinase inhibitor does inhibit thrombin (Rimon *et al.*, 1966; Lane *et al.*, 1975), but some have suggested that it does not (Heimbürger *et al.*, 1971; Learned *et al.*, 1976). When only short times (5 min) of incubation of thrombin and α_1 -proteinase inhibitor have

been used, the conclusion has been that α_1 -proteinase inhibitor is not an inhibitor of thrombin. The molar ratio of α_1 -proteinase inhibitor to thrombin would have to be very high for an effect on thrombin to be visible in 5 min, since this inhibition is a time-dependent process. It is difficult to determine the actual molar ratio used by Learned *et al.* (1976), since it appears from the data given that both the α_1 -proteinase inhibitor and thrombin were only partially purified. For an unambiguous answer, highly purified proteins should have been used. In the present study, homogeneous preparations of human thrombin and human α_1 -proteinase inhibitor were used, and the data clearly show that thrombin is inhibited in the presence of α_1 -proteinase inhibitor and that this inhibition cannot be due to a contaminant of the α_1 -proteinase-inhibitor preparation, such as proteinases or antithrombin III. Unlike the interaction of trypsin and α_1 -proteinase inhibitor, the inhibition of thrombin clotting and esterolytic activities proceeds at a measurable rate and also in proportion to the ratios of the two proteins.

All of the α_1 -proteinase-inhibitor interactions previously studied have been demonstrated to be due to the formation of a stable complex with the proteinase. As the proteinase/(α_1 -proteinase inhibitor) molar ratio is increased, increasing amounts of complex appear, and proteinase and α_1 -proteinase inhibitor bands disappear in disc-gel electrophoresis (Travis *et al.*, 1974). Complex-formation is complete at a proteinase/(α_1 -proteinase inhibitor) molar ratio of 1:1 (Baugh & Travis, 1976). Thrombin and α_1 -proteinase inhibitor also form a complex, essentially complete at a 1:1 molar ratio, as shown by both sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis and Sephadex G-75 chromatography. Protection of activity by substrate and lack of complex-formation with di-isopropyl phosphorofluoridate-treated thrombin indicate that the active site of thrombin is involved in its inactivation and in complex-formation. Loss of effect on thrombin by modification of lysine residues in α_1 -proteinase inhibitor indicates that its reactive centre is involved in the inactivation of thrombin.

The formation of a 1:1 complex of thrombin and α_1 -proteinase inhibitor may be of physiological importance, since the concentration of α_1 -proteinase inhibitor in blood is much higher than the maximum possible concentration of thrombin. The mechanism may be different from that involving trypsin, however, since α_1 -proteinase inhibitor is capable of inactivating thrombin to a greater degree at high thrombin/(α_1 -proteinase inhibitor) molar ratios than it is capable of doing with trypsin. The nature of the mechanism by which α_1 -proteinase inhibitor inactivates thrombin has not yet been determined.

It is not known what role α_1 -proteinase inhibitor might play in the regulation of coagulation and

fibrinolysis. α_1 -Proteinase inhibitor is best known for its ability to inhibit trypsin and chymotrypsin, although neither of these proteinases is found in blood. The results demonstrated in the present paper, as well as the fact that this protein can also inactivate plasmin (Heimburger *et al.*, 1971; K. Beatty & J. Travis, unpublished work), justify continued investigation of the function of α_1 -proteinase inhibitor in these two systems.

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