Inhibition of the Thrombin–Fibrinogen Reaction by a Macromolecular Factor from Rat Liver

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1. The supernatant obtained by centrifugation of a rat liver homogenate at 100000g for 1 h contained a heat-labile macromolecular inhibitor of the thrombin–fibrinogen reaction. 2. The inhibitor was purified to electrophoretic homogeneity by repeated preparative polyacrylamide disc electrophoresis. Inhibition was observed with purified inhibitor equivalent to about 1 µg of protein/ml. 3. The inhibitor had a *pI* of 3.50–3.75, a molecular weight (from sodium dodecyl sulphate–polyacrylamide-gel electrophoresis) of 72000 ± 3000 and was inactivated by *p*-hydroxymercuribenzoate or 5,5'-dithiobis-(2-nitrobenzoic acid). 4. Kinetic studies revealed a non-competitive inhibition, with the inhibitor probably acting on the thrombin–fibrinogen complex.

Studies on the subcellular localization of the coagulation factor VII (Gaarder & Prydz, 1967, 1969) revealed the presence of an inhibitor of blood coagulation in the 100000g supernatant from perfused rat liver. We report here the purification of this inhibitor and some studies of its mode of action.

Materials and Methods

Special chemicals and biochemicals

Trypsin was from Difco, Detroit, Mich., U.S.A.; 5,5'-dithiobis-(2-nitrobenzoic acid) was from Aldrich, Milwaukee, Wis., U.S.A.; *p*-hydroxymercuribenzoate and dithioerythritol were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; heparin was from Apotekernes Laboratorium, Oslo, Norway; *p*-tosyl-L-arginine methyl ester was from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.

Substrates for testing proteolytic activity

Casein (Eastman Kodak, Rochester, N.Y., U.S.A.) was dissolved in 0.075M-NaCl in 0.025M-sodium phosphate buffer, pH 7.6, heated to 85–90°C, dialysed against double-distilled water and diluted with VBS medium,* pH 7.4, to E_{280} 0.7. Other substrates were ovalbumin (Sigma), human albumin (grade III; Sigma), azo-fibrinogen (bovine; Calbiochem A.-G., Lucerne, Switzerland) and azo-collagen (Calbiochem), all at 1 mg/ml in VBS medium.

Coagulation reagents

Bovine thrombin (Topostasine; Hoffmann–La Roche and Co. Ltd., Basle, Switzerland) was dis-

solved in VBS medium to 6 units/ml (as indicated by the manufacturer) if not otherwise stated.

Reptilase (Kramer, Lausanne, Switzerland) was used as obtained.

Human fibrinogen (Kabi, Stockholm, Sweden) was dissolved to a final concentration of 3.3 mg/ml in 0.3M-NaCl in 28.5 mM-veronal buffer, pH 7.3, if not otherwise stated. A standard curve for the thrombin–fibrinogen reaction was established with this fibrinogen concentration.

Preparation of inhibitor

The 100000g supernatants were prepared from perfused livers of starved male rats (Charles River, Wilmington, Mass., U.S.A., or Wistar/Møll, Møllgaard, Havrup, Denmark). Each rat was given an intraperitoneal injection of warfarin [3-(α -acetylbenzyl)-4-hydroxycoumarin; Marevan; Evans Medical Ltd., Speke, Liverpool, U.K.] (5 mg/100 g body wt.) 28 and 4 h before being killed to decrease the amounts of vitamin K-dependent procoagulants (factors II, VII, IX and X) in the supernatants and thus prevent generation of thrombin. Supernatants from untreated rats gave similar results. The livers were perfused and washed with solution A (0.25M-sucrose–1 mM-EDTA–35 mM-tris–HCl, pH 7.8) before being minced and homogenized in solution B (0.25M-sucrose–25 mM-KCl–10 mM-MgCl₂–35 mM-tris–HCl, pH 7.8). The homogenate was centrifuged at 1500g for 2–3 min and the supernatant was centrifuged at 100000g for 1 h. The 100000g supernatant was stored in 5 ml portions at –20°C.

Three types of inhibitor preparations were used (type I, 100000g supernatant; type II, 100000g supernatant purified by filtration through Bio-Gel P-100; type III, 100000g supernatant purified by preparative disc electrophoresis).

* Abbreviation: VBS medium, veronal-buffered saline (9 vol. of 0.15M-NaCl, 1 vol. of 28.5 mM-veronal buffer) (Owren, 1947).

Coagulation tests

These were performed at 37°C. The reagents were preincubated for 3 min before addition of the final reagent unless otherwise stated.

The thromboplastin system contained: normal citrated human plasma, 0.1 ml; VBS medium, 0.1 ml; tissue thromboplastin (Hjort, 1957), 0.1 ml; 30 mM-CaCl₂, 0.1 ml.

The thrombin-fibrinogen reaction system contained: fibrinogen solution, 0.2 ml; VBS medium, 0.1 ml; thrombin solution, 0.1 ml.

The reptilase-fibrinogen reaction system contained: fibrinogen solution (6.7 mg/ml), 0.2 ml; VBS medium, 0.1 ml; 30 mM-CaCl₂, 0.1 ml; reptilase (undiluted), 0.1 ml.

Fibrin monomer was prepared essentially as described by Donnelly *et al.* (1955). Fibrinogen was clotted once with thrombin and the clot was dissolved to a final concentration of 8.4 mg/ml.

The fibrin-polymerization system contained: VBS medium, 0.1 ml; 0.013 M-glycine-NaOH buffer (pH 10.5), 0.1 ml; fibrin monomer solution (pH 5.3), 0.1 ml.

The final pH in the reaction mixture was 7.5.

Inhibitor activity

This was tested by substituting inhibitor solutions for VBS medium in the above systems. Inhibitor solutions were always dialysed against VBS medium before testing. All dilutions for testing were done in this buffer. An inhibitor solution was considered to have 1 unit/ml when it decreased the activity of the standard thrombin solution by 0.06 N.I.H. unit/ml in the thrombin test system described above.

Inhibition of thrombin was also tested with *p*-tosyl-L-arginine methyl ester as substrate by incubating 1 ml of 0.1 M-*p*-tosyl-L-arginine methyl ester in 28.5 mM-veronal buffer (Owren, 1947) adjusted to pH 7.2, 2 ml of inhibitor solution (type III) and 2 ml of VBS medium with thrombin (final concn. 5 N.I.H. units/ml). The mixture was kept at pH 7.2 with 0.01 M-KOH by means of a Radiometer TTT 1c pH-stat, and the volume used was compared with that used in controls without inhibitor.

Heparin cofactor activity

This was tested in the following systems. (a) Incubation with thrombin: 0.2 ml of thrombin (10 N.I.H. units/ml); 0.2 ml of inhibitor (type II); 0.2 ml of heparin (25 units/ml); 0.3 ml of fibrinogen. (b) Incubation with fibrinogen: 0.8 ml of fibrinogen; 0.2 ml of inhibitor (type II); 0.2 ml of heparin; 0.1 ml of thrombin (10 N.I.H. units/ml).

Standard curves were established by substituting VBS medium for heparin and inhibitor. The inhibi-

tion was compared with the sum of that obtained in separate incubations with inhibitor or heparin.

Caseinolytic activity

This was assayed as described by Jacobsen (1964). Proteolytic activity was also tested by using ovalbumin, human albumin, azo-fibrinogen and azo-collagen as substrates in the same system, except that lysis of the azo-collagen was monitored at 520 nm.

Anti-trypsin activity

This was investigated either by incubating 2 ml of the various substrates for proteolysis with 0.2 ml of trypsin solution (2.5 mg/ml in VBS medium) and 0.2 ml of inhibitor solution (type II), or with *p*-tosyl-L-arginine methyl ester as substrate for esterolysis: 3 ml of trypsin solution 1 ml of 0.2 M-*p*-tosyl-L-arginine methyl ester in 0.15 M-KCl, adjusted to pH 7.0 with KOH, and 2.7 ml of inhibitor (type II) were mixed and incubated at 37°C. The amount of 0.01 M-KOH used to titrate the mixture to pH 7.0 was measured by a Radiometer TTT 1c pH-stat.

Other procedures

Preparative disc electrophoresis (Buchler Instruments, Fort Lee, N.J., U.S.A.), gel filtration (on Bio-Gel P-100 (Bio-Rad, Richmond, Calif., U.S.A.) and Sepharose 4B (Pharmacia, Uppsala, Sweden)) and electrofocusing (LKB, Stockholm, Sweden) were carried out as described by the manufacturers. A slight modification of the method of Grossbach (1965) was used for analytical disc electrophoresis. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis was carried out as described by Glossmann & Lutz (1970). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard. All buffers were made from double-distilled water.

Results

In preliminary experiments the 100000g supernatant inhibited the coagulation of whole plasma with tissue thromboplastin as well as the thrombin-fibrinogen reaction. The inhibitory activity was heat-labile and was recovered in the excluded fraction from Sephadex G-25. It was not adsorbed on BaSO₄ or glass. The inhibitor of the thrombin-fibrinogen reaction was also found in supernatants from unperfused bovine liver. The 100000g supernatant had considerable general proteolytic activity. Purified fractions (types II and III) had no proteolytic activity on any of the substrates tested and such preparations were used for the subsequent investigations.

Table 1. *Typical results of purification of the inhibitor from rat liver supernatant by using a variety of procedures*

Crude supernatant was the starting material in each experiment except for the repetition of preparative disc electrophoresis.

	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Purification	Recovery (%)
Crude supernatant	35–52	27–40	1.1–1.6		100
(NH ₄) ₂ SO ₄ precipitation	23.3	14.5	1.6	1.3	87.3
Gel filtration (Bio-Gel P-100)	48.2	1.4	34.4	26.7*	409*
Batch adsorption (CM-Sephadex C-50)	16.0	5.35	3.0	2.8	94.8
Precipitation at pH 5.15	30.5	9.34	3.2	2.0	125*
Preparative disc electrophoresis	48.7	0.027	1805	1120	85.5
Preparative disc electrophoresis (repeated)	38.8	0.0095	3790	2350	69.5

* Probably due to removal of procoagulant material.

Purification of inhibitor

Gel filtration. A Bio-Gel P-100 column (3cm × 55cm) was equilibrated and eluted with VBS medium at a flow rate of about 20ml/h. The inhibitor was eluted just before haemoglobin. A 27-fold purification was obtained (Table 1), probably partly due to removal of procoagulant material, which counteracted the inhibitor in the crude supernatant. Such procoagulant material was recovered in later fractions.

Ammonium sulphate precipitation. The inhibitor was precipitated at between 20 and 60% saturation. A slight purification was obtained (Table 1).

Batch adsorption with CM-Sephadex. CM-Sephadex C-50 (Pharmacia) was equilibrated with 0.01M-sodium phosphate buffer, pH 6.5. Gel (3ml) and 2ml of 100000g supernatant were stirred for 2h at 2°C. This left the inhibitor unadsorbed and gave about three-to-four-fold purification (Table 1).

Precipitation at pH 5.15. Equal volumes of 0.2M-sodium maleate buffer, pH 5.0, and inhibitor solution were mixed, giving pH 5.15. After precipitation and dialysis at 4°C the supernatant was tested for inhibitory activity. A twofold purification was obtained.

Preparative disc electrophoresis. This gave 800–1000-fold purification (Table 1). The inhibitor was eluted immediately before the tracking dye (Bromophenol Blue). Preparations obtained after two successive runs gave one band in analytical disc electrophoresis at pH 8.6 and had a specific activity of about 3100–3800 units/mg of protein (Table 1).

Physical characteristics of the purified inhibitor

From gel-filtration results a molecular weight of 70000–80000 was estimated. In sodium dodecyl sulphate–polyacrylamide-gel electrophoresis a single band with a molecular weight of 72000 ± 3000 was observed. The *pI* was determined to be 3.50–3.75 by isoelectric focusing. The absorbance at 450nm excluded the presence of bilirubin, which has been

reported to exhibit antithrombin activity (Kopeć *et al.*, 1961).

Stability

The main problem in purifying the inhibitor was its lability. The activity was fairly stable at 4°C for up to 20h in the range pH 6.5–10, but decreased during longer dialysis and various other treatments. The activity was destroyed by heat (65°C/30min) and by *p*-hydroxymercuribenzoate (50 µg/ml) or 5,5'-dithiobis-(2-nitrobenzoic acid) (4.6mM). Ca²⁺ and Mg²⁺ (2mM) did not influence the inhibition, nor did they offer any protection.

Some protection of the inhibitory activity was observed with EDTA (2mM) and dithioerythritol (1mM). EDTA is in itself an inhibitor of the thrombin–fibrinogen reaction (Godal, 1960; Bithell, 1964). The activities of the liver inhibitor and EDTA were additive. The apparent protection of the inhibitor might be due to incomplete removal of EDTA in spite of extensive dialysis before testing.

Inhibitor activity

The purified inhibitor had no detectable anti-trypsin activity and no proteolytic or esterolytic activity. The inhibitor did not progressively inhibit thrombin, as preincubation with thrombin or fibrinogen gave essentially the same inhibition and no increase compared with uncubated controls. No inhibition of the esterolytic activity of thrombin on *p*-tosyl-L-arginine methyl ester was found.

The inhibitor did not influence the reptilase time nor the polymerization of fibrin monomer induced by adjustment of pH in monomer solutions (Donnelly *et al.*, 1955).

We looked for complex-formation between inhibitor and fibrinogen by displacement of the peak of

inhibitor when co-chromatographed with fibrinogen on Sepharose 4B (gel bed 3cm×39cm) in VBS medium, but no change was observed. The inhibitor was recovered in active form after the same elution volume as when it was chromatographed without fibrinogen.

In the tests for heparin cofactor activity a slight synergistic effect was obtained with heparin and inhibitor in preincubation with thrombin, whereas an antagonistic effect was observed in preincubation with fibrinogen (Table 2).

Thrombin–fibrinogen reaction

The velocity of the thrombin–fibrinogen reaction was measured by the reciprocal value of the coagulation time, i.e. the sum of the time required for thrombin to build up a concentration of fibrin monomer sufficient to transform the solution into a gel (the enzyme phase: step I) and the time required for this transformation (the polymerization phase: step II). This measurement is probably valid with low thrombin concentration, i.e. when step I is rate-limiting. At thrombin concentrations above 1–2 units/ml (with 1–2mg of fibrinogen/ml) a plot of velocity versus thrombin concentration is no longer linear (Fig. 1), probably because step II becomes rate-limiting. To secure a linear relation between thrombin concentration and reaction velocity (the inverse of coagulation time) in all kinetic studies the concentrations of thrombin and fibrinogen were between 0.75 and 1.0 N.I.H. unit/ml and 0.125–3.3 mg/ml respectively, if not otherwise indicated.

A saturation plateau was obtained in Michaelis–Menten plots at fibrinogen concentrations of about

0.25–1.7 mg/ml with 1 unit of thrombin/ml (Fig. 2), whereas higher substrate concentrations gave a slight inhibition. K_m for the thrombin–fibrinogen reaction was about 9.4×10^{-7} M.

Kinetics of inhibition

Maximum inhibition obtained with purified inhibitor was about 70% (Fig. 3). In separate experiments purified inhibitor at a final concentration of 7.5 µg/ml decreased the thrombin activity by 40%. This inhibition was not overcome by increasing the thrombin concentration tenfold or the fibrinogen concentration fivefold. On the contrary, increasing amounts of substrate (up to a final concentration of about 0.3 mg/ml) increased the inhibition when 0.75 N.I.H. unit of thrombin and 7.5 µg of inhibitor/ml were used.

Fig. 1. Effect of increasing thrombin concentration on the velocity of the thrombin–fibrinogen reaction

For experimental details see the text. The final concentration of fibrinogen was 3.3 mg/ml.

Table 2. Effect of inhibitor and heparin on the thrombin–fibrinogen reaction

A, Preincubation of inhibitor or heparin or both with thrombin (3 min at 37°C) before mixing with fibrinogen solution. Final concentrations were: thrombin, 0.83 N.I.H. unit/ml, fibrinogen 2.5 mg/ml, heparin 2.1 units/ml, inhibitor 170 µg/ml. B, Preincubation of inhibitor or heparin or both with fibrinogen (3 min at 37°C) before mixing with thrombin solution. Final concentrations were: thrombin 1.5 N.I.H. units/ml, fibrinogen 1.65 mg/ml, heparin 3.1 units/ml, inhibitor 170 µg/ml.

System	Inhibition (%)		
	Heparin	Inhibitor (type II)	Inhibitor + heparin
A	23	25	69
B	61	35	78

Fig. 2. Michaelis–Menten plot of the velocity of the thrombin–fibrinogen reaction at a constant thrombin concentration

For experimental details see the text. The final thrombin concentration was 1 N.I.H. unit/ml.

A double-reciprocal plot (Fig. 4) confirmed that the inhibitor was non-competitive. From a similar reciprocal plot with various inhibitor concentrations the intercepts on the ordinate were plotted against the

Fig. 3. Effect of purified inhibitor in the thrombin-fibrinogen system

For experimental details see the text. The final concentration of thrombin was 0.75 N.I.H. unit/ml, and of fibrinogen 0.51 mg/ml.

inhibitor concentration (Fig. 5). The hyperbolic plot was in accordance with the results obtained when complete inhibition of a small amount of thrombin was attempted. A plot of velocity versus thrombin concentration at various fixed inhibitor concentrations went through the origin (Fig. 6). A slight change in K_m was found. The inhibitor constant K_i was $2.5 \times 10^{-7} M$, calculated from the Lineweaver-Burk plots.

Discussion

Previous workers have described various coagulation inhibitors obtained from liver (Fritz *et al.*, 1968; Werle *et al.*, 1967). Deykin *et al.* (1969) found a competitive inhibitor of activated (by Russell's-viper venom) factor X in bovine liver. Their results also showed a marked inhibition of the thrombin-fibrinogen reaction by the supernatant, but the two activities were partially separated by $(NH_4)_2SO_4$ precipitation. Other substances in liver inhibiting proteases in general and thus also inhibiting blood coagulation (Fritz *et al.*, 1967, 1968; Werle *et al.*, 1967) had a molecular weight of about 6500 and are clearly different from the inhibitor described here. To our knowledge a macromolecular inhibitor of the thrombin-fibrinogen reaction without general anti-proteolytic activity has not been described from liver. The inhibitor is a heat-labile macromolecule with a

Fig. 4. Double-reciprocal plot of reaction velocity of the thrombin-fibrinogen reaction versus fibrinogen concentration at constant thrombin concentration

For experimental details see the text. The thrombin concentration was 0.75 N.I.H. unit/ml. ○, Control; ●, inhibitor (final concn. 7.5 μg/ml).

Fig. 5. Intercept on ordinate from a double-reciprocal plot of the thrombin-fibrinogen reaction at various inhibitor concentrations plotted versus the inhibitor concentrations

For experimental details see the text. Inhibitor solution type II was used.

molecular weight of about 72000 and a low isoelectric point (3.5–3.7). This may explain the migration ahead of the tracking dye in polyacrylamide-gel electrophoresis. Its activity can be observed with concentrations down to $1 \mu\text{g/ml}$ in a test system with 1 unit of thrombin/ml and 1 mg of fibrinogen/ml. One or more thiol groups were necessary since 5,5'-dithiobis-(2-nitrobenzoic acid) and *p*-hydroxymercuribenzoate abolished and dithioerythritol protected the inhibitory activity. Oxidation of essential thiol groups may also at least partially explain the instability. No bivalent ions appeared necessary.

The inhibitor had no proteolytic nor anti-trypsin activity. Unlike antithrombin III, its effect on the thrombin-fibrinogen reaction was not time-dependent. In the presence of heparin a slight synergistic effect was observed, but much less than that found when heparin and anti-thrombin III were used (Abildgaard, 1968). We therefore conclude that the inhibitor is different from the previously known macromolecular inhibitors of the thrombin-fibrinogen reaction, like anti-thrombin III, α_2 -macroglobulin and α_1 -trypsin inhibitor.

The kinetic study of inhibitor action in the thrombin-fibrinogen reaction necessitated a careful selection of thrombin and fibrinogen concentrations. Too little fibrinogen gave a reaction of higher than zero-order, too much fibrinogen gave substrate inhibition. Too much thrombin invalidated the velocity measurements, since step II (the polymerization

Fig. 6. Effect of inhibitor at low thrombin concentration

For experimental details see the text. The final concentration of fibrinogen was 0.51 mg/ml . ○, Control; ●, final concn. of inhibitor $3.5 \mu\text{g/ml}$; △, final concn. of inhibitor $7 \mu\text{g/ml}$.

of fibrin monomer) became rate-limiting. Even with the appropriate concentrations the assumption that the coagulation time is a direct function of $1/v$ is not proved. The kinetic data obtained will require confirmation by direct measurements of the velocity of the thrombin-fibrinogen reaction in the presence of inhibitor.

The K_m for the thrombin-fibrinogen reaction was about $9.4 \times 10^{-7} \text{ M}$. Shinowara (1965) reported K_m $4.38 \times 10^{-7} \text{ M}$ for the reaction of human thrombin with human fibrinogen, and the slightly higher values reported here with bovine thrombin and human fibrinogen may be due to the species difference. From the results of Blombäck (1958) and Abildgaard (1967) on the appearance of *N*-terminal glycine, K_m values of $0.9 \times 10^{-5} \text{ M}$ and $0.8 \times 10^{-5} \text{ M}$ have been calculated. On the basis of the release of *N*-terminal glutamic acid with the A peptide of bovine fibrinogen, Blombäck (1967) calculated a K_m of $1.2 \times 10^{-5} \text{ M}$. The results of Abildgaard (1967) were obtained with human thrombin and fibrinogen, whereas Blombäck (1967) used a bovine system.

The eight- to ten-fold difference between the K_m values calculated from the various *N*-terminal measurements and those based on coagulation-time determinations remains unexplained. The *N*-terminal measurements involve certain correction factors that may influence the K_m calculations. On the other hand, although the experiments were carried out in the concentration range where a plot of the inverse coagulation time versus thrombin concentration was linear, the relation assumed between the coagulation time and the inverse of the reaction velocity has not been directly proved. This assumption is obviously important for the calculation of K_m .

The thrombin-fibrinogen reaction proceeds in several steps. The inhibitor might act in one or more

of the following ways: (a) complex-formation with thrombin; (b) complex-formation with fibrinogen; (c) complex-formation with the thrombin-fibrinogen complex; (d) complex-formation with fibrin monomer.

The inhibitor was non-competitive with respect to both enzyme and substrate. The inhibitor probably did not act on fibrinogen or fibrin, since it influenced neither polymerization nor reptilase time and no complex-formation with fibrinogen was detected. An effect on thrombin appears to be unlikely, since no inhibition of the esterolysis of *p*-tosyl-L-arginine methyl ester by thrombin was found, and increasing doses of thrombin did not overcome the inhibition.

Interaction of the inhibitor with the thrombin-fibrinogen complex or the acylated thrombin intermediate remains the most likely mechanism. The inhibitor had a low *pI* and consequently a high negative charge at physiological pH values. In this respect it is similar to heparin, which also has been suggested to interact with the thrombin-fibrinogen complex (Abildgaard, 1968). The absence of inhibition of the reptilase activity was another similarity.

The reaction did proceed in the presence of inhibitor, even when small doses of thrombin were used (Fig. 7), indicating that the enzyme-substrate-inhibitor complex broke down, but at a slower rate than the enzyme-substrate complex. For this type of non-competitive inhibition, the reciprocal plots do not intersect on the $1/s$ axis; a hyperbolic curve is expected when the intercepts of double-reciprocal plots on their ordinates are plotted against the inhibitor concentration, and on increasing the substrate concentration increasing inhibition would be expected.

Such kinetics were observed for the present inhibitor (Figs. 4 and 5). We conclude that the inhibitor probably interacted with the enzyme-substrate complex.

The physiological significance of this inhibitor remains unknown. Unexplained coagulation disturbances are known to occur in patients with liver disease. The presence of an inhibitor in human liver and plasma similar to that described here might contri-

bute to such disturbances. Nothing is presently known about the influence of the inhibitor on the thrombin-induced platelet aggregation.

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