Biochem. J. (1987) 242, 881–887 (Printed in Great Britain)

Inhibited thrombins

Interactions with fibrinogen and fibrin

Marek KAMINSKI and Jan McDONAGH

Departments of Pathology, Beth Israel Hospital and Harvard Medical School, and the Charles A. Dana Research Institute, Beth Israel Hospital, Boston, MA 02215, U.S.A.

Fibrin-monomer–Sepharose was used to study thrombin binding to fibrin and the role of the enzyme active centre in this interaction. Binding properties of preformed enzyme–inhibitor complexes, as well as inhibition of thrombin already adsorbed to fibrin monomer, were investigated. No apparent difference was found in binding properties of phenylmethanesulphonyl fluoride-, D-Phe-Pro-Arg-CH_2Cl- and dansylarginine *NN*-(3-ethylpentane-1,5-diyl)amide-inhibited thrombins. Also, the elution profile of phenylmethanesulphonyl fluoride-inhibited thrombin from fibrinogen–Sepharose was identical with that of active thrombin from fibrin-monomer–Sepharose. Thus far the only low-M_r inhibitor that prevents thrombin from binding to fibrin monomer is pyridoxal 5′-phosphate. Preformed hirudin–thrombin complexes do not interact with fibrin. The extent to which the active centre of thrombin associated with fibrin is still accessible to substrates and inhibitors was also studied. Thrombin bound to fibrin hydrolyses a synthetic substrate at the same rate as the free enzyme. Water-soluble low-M_r inhibitors such as D-Phe-Pro-Arg-CH_2Cl and dansylarginine *NN*-(3-ethylpentane-1,5-diyl)amide can readily modify the active centre of the fibrin-associated enzyme, and the active centre is exposed to the degree that displacement of dansylarginine *NN*-(3-ethylpentane-1,5-diyl)amide by D-Phe-Pro-Arg-CH_2Cl is possible without disturbing the binding. Hirudin disrupts the affinity between thrombin and fibrin. These data indicate that the active centre of thrombin associated with fibrin through extended binding is fully exposed and freely accessible. It is possible that extended binding may play a regulatory role in the activation of Factor XIII by thrombin, as well as inactivation of this enzyme by antithrombin III.

INTRODUCTION

Interaction between thrombin (EC 3.4.21.5) and fibrinogen is a major event in the series of finely controlled reactions of the coagulation cascade. It results in activation of fibrinogen to fibrin monomers, which then spontaneously polymerize to form a three-dimensional fibrin network. Thrombin specifically cleaves fibrinopeptides A and B from the *N*-terminal ends of the Aα and Bβ chains of fibrinogen. Cleavage of fibrinopeptide A is a prerequisite for fibrin-monomer self-assembly, and release of fibrinopeptide B normally occurs after initiation of polymerization (Blombäck *et al.*, 1978). Residues 11–25 of the Aα chain of fibrinogen appear to contain a thrombin-binding domain; but other, long-range, interactions have also been described (Nagy *et al.*, 1982; Marsh *et al.*, 1982).

Two different approaches have been used to investigate the interaction of thrombin with its product, fibrin. In a study where fibrin clots were equilibrated in buffer and diffusion of ^{125}I-thrombin was monitored, two classes of binding sites were reported (Liu *et al.*, 1979). In other studies, flow conditions through columns containing fibrin-monomer–Sepharose or fibrin gel were employed in order to define the interaction between thrombin and fibrin monomer compared with fibrin polymer. A single class of binding sites was observed with fibrin monomer, and the binding was inversely related to the ionic strength of the test system. With both test systems (static and freely flowing), a K_a of approx. 5×10^5 was obtained (Liu *et al.*, 1979; Kaminski & McDonagh, 1983). Increasing ionic strength releases thrombin from its binding domain on fibrin monomers (Kaminski & McDonagh, 1983; Berliner & Sugawara, 1985). High $CaCl_2$ specifically disrupts thrombin–fibrin-monomer binding, even at physiological ionic strength. Furthermore, polymerization of fibrin monomers also disrupts fibrin-monomer affinity for thrombin and results in the release of thrombin into the fluid phase of the fibrin gel (Wilner *et al.*, 1981; Fenton *et al.*, 1979; Carney *et al.*, 1979). However, some of it can remain attached to the branching point of the clot (Liu *et al.*, 1985). Thrombin was also found to interact with fragment X of fibrinogen (Liu *et al.*, 1981) and to be recovered from the clot upon plasmic digestion (Francis *et al.*, 1981). Thrombin affinity to fibrin has also been found to involve an anionic binding site (Berliner & Sugawara, 1985), independent of the immediate catalytic centre (Kaminski & McDonagh, 1982; Fenton *et al.*, 1981; Wilner *et al.*, 1981), and to have a regulatory effect on the rates of fibrinopeptide release and fibrin polymerization at low ionic strength (Kaminski & McDonagh, 1985; Carr *et al.*, 1985).

In the present study the active centre of thrombin has been probed with specific inhibitors under test conditions in which the enzyme was bound to fibrin monomer.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; DAPA, dansylarginine *NN*-(3-ethylpentane-1,5-diyl)amide; PPACK, D-Phe-Pro-Arg-CH_2Cl; S-2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride.

Equilibrium binding properties of preformed enzyme–inhibitor complexes, as well as inhibition of thrombin already adsorbed to fibrin monomer, were investigated. In addition, the tripeptide *p*-nitroanilide thrombin substrate, S-2238, was used to test the functional integrity and accessibility of the active centre while the enzyme was bound to fibrin monomer.

EXPERIMENTAL

Materials

Fibrinogen–Sepharose and fibrin-monomer–Sepharose were prepared as previously described (Heene & Matthias, 1973). Fibrin-monomer–Sepharose was used for both column chromatography and batch absorption. For experiments shown in Fig. 1 and 2, the gel was equilibrated with 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.4, and final elution of bound thrombin was achieved with the same buffer containing 50 mM-$CaCl_2$. To increase the affinity between thrombin and fibrin monomer in the remaining experiments, buffer of lower ionic strength, 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4, was used for equilibration; elution was carried out with 100 mM-$CaCl_2$ in the same buffer.

Preparation of ^{125}I-labelled thrombin

Purified human α-thrombin (specific activity 2207 NIH units/mg) was generously provided by Dr. John Fenton II (New York State Department of Health). It was radiolabelled by the lactoperoxidase/glucose oxidase procedure and further purified on fibrin-monomer–Sepharose affinity columns. Clotting times were measured as described by Kaminski & McDonagh (1983). Radiolabelled thrombin retained its full clotting activity.

Inhibition of thrombin with PMSF

^{125}I-labelled thrombin (1200 μl) diluted with unlabelled enzyme (final concn. 110 μg/ml) in 0.05 M-Tris/HCl/0.1 M-NaCl, pH 8.1, was mixed with 200 μl of freshly prepared PMSF (Sigma) in methanol (3 mg/ml), or with 200 μl of methanol only, and incubated at room temperature for 30 min. Enzyme mixed with methanol retained its clotting activity; with the inhibited enzyme no activity was detected in 30 min. To both reaction mixtures 1600 μl of equilibration buffer was added, and 1400 μl of each solution was applied on the affinity columns.

Modification of thrombin with pyridoxal 5′-phosphate

Phosphopyridoxylation of ^{125}I-labelled thrombin was kindly performed by Dr. Michael J. Griffith (University of North Carolina at Chapel Hill) with heparin present in the reaction mixture (Griffith, 1979). The modified enzyme retained about 15% of its original clotting activity.

Water-soluble inhibitors and synthetic substrate

Hirudin was obtained from Sigma, PPACK was from Calbiochem–Behring, DAPA was kindly provided by Dr. Kenneth G. Mann, University of Vermont, and by Dr. Jacek Hawiger, New England Deaconess Hospital, and S-2238 was from Kabi Vitrum. These reagents were dissolved in 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4. When ^{125}I-thrombin was made to react with PPACK or hirudin under the conditions described here, no clotting activity remained.

Fluorescence measurements

These were performed in a Perkin–Elmer 650-105 fluorescence spectrometer, with excitation at 335 nm and emission at 565 nm.

RESULTS

Binding of PMSF- and pyridoxal 5′-phosphate-inhibited thrombins to fibrinogen- and fibrin-monomer–Sepharose

When PMSF-inhibited thrombin was applied to a fibrin-monomer–Sepharose column and then washed with 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.4, no unbound radioactivity was eluted, indicating that all of the applied material was adsorbed on the column (Fig. 1). Introduction of 50 mM-$CaCl_2$ resulted in immediate elution of 85–95% of the applied radioactivity. Exactly the same pattern of elution was observed when active thrombin was used, and the eluted peak contained activity coincident with the radioactivity. The same elution profile was also observed when PMSF-inhibited ^{125}I-labelled thrombin was chromatographed on fibrinogen–Sepharose.

Inhibition of thrombin with pyridoxal 5′-phosphate resulted in a different elution pattern from fibrin-monomer–Sepharose (Fig. 2). Washing the column with sample buffer resulted in appearance of a radioactivity peak in the void volume, which contained approx. 85% of the enzyme applied. Addition of 50 mM-$CaCl_2$ to the buffer resulted in elution of a second radioactivity peak

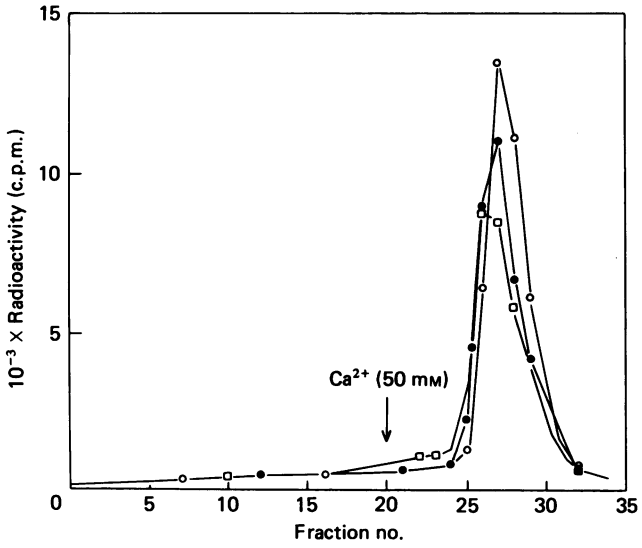

Fig. 1. PMSF-inhibited thrombin and active thrombin: binding to fibrinogen- and fibrin-monomer–Sepharose

Two columns containing fibrin-monomer–Sepharose and one with fibrinogen–Sepharose (2 ml; 16 mg of fibrinogen or fibrin/ml of settled gel) were equilibrated with 0.05 mM-Tris/HCl/0.1 M-NaCl, pH 7.4. Active or PMSF-inhibited thrombin (51 μg) in the same buffer (1400 μl) was applied to each fibrin-monomer–Sepharose column, and PMSF-inhibited thrombin to the fibrinogen-Sepharose column. At fraction 20, elution buffer containing 50 mM-$CaCl_2$ was introduced. Fraction volume = 360 μl; flow rate = 200 μl/min. Symbols represent fibrin monomer–Sepharose with PMSF-inhibited thrombin (●) or active thrombin (○), and fibrinogen–Sepharose with PMSF-inhibited thrombin (□).

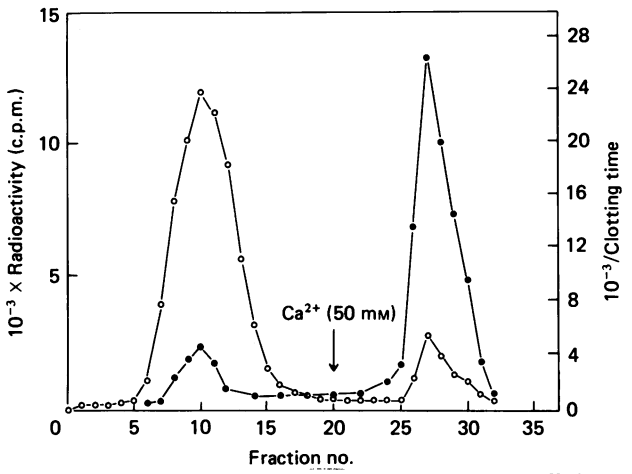

Fig. 2. Pyridoxal 5'-phosphate-modified thrombin: affinity to fibrin monomer

A mixture of unlabelled and ^{125}I-labelled thrombin was treated with pyridoxal 5'-phosphate in the presence of heparin; the reaction product retained about 15% of its initial clotting activity. Treated enzyme (17 μg) in 1 ml of 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.4, was applied on a fibrin-monomer–Sepharose column (2 ml of gel; 16 mg of fibrin/ml) and the column was washed with the same buffer. Fractions were collected as in Fig. 1. At fraction 20, elution with buffer containing 50 mM-$CaCl_2$ was begun. Fibrinogen-clotting activity (●) and radioactivity (○) were measured in each fraction.

containing the remaining enzyme. Measurement of clotting times in each fraction showed that nearly all the clotting activity was limited to the second peak. Hence only the fraction of thrombin molecules that retained clotting activity bound to fibrin monomer; the fraction that had lost clotting activity through phosphopyridoxylation also lost ability to bind to fibrin monomer.

PPACK, hirudin and DAPA: effects on thrombin binding to fibrin monomer

Hirudin represents a class of high-M_r protein inhibitors of thrombin (M_r 7000), with a high affinity. As shown in Fig. 3, chromatography of preformed thrombin–hirudin complexes on fibrin-monomer–Sepharose columns under normal elution conditions resulted in appearance of all the radioactivity in the void volume, indicating that no interaction between the thrombin–hirudin complex and fibrin monomer had occurred. When active thrombin was applied first and then, after 20 fractions were collected, hirudin was added to the elution buffer, a radioactive peak containing all the enzyme but no enzymic activity immediately began to be eluted. This indicates that adsorption to fibrin did not protect thrombin from interaction with this high-M_r inhibitor.

Fig. 3 shows that modification of the active-centre histidine with PPACK, which resulted in total loss of thrombin activity toward its natural and synthetic substrates, did not prevent its specific interaction with fibrin monomer. The presence of $CaCl_2$ in the washing buffer was required to remove this modified enzyme from the column. PPACK could also react with the active centre of thrombin already bound to fibrin monomer, but this did not affect its elution profile. $CaCl_2$ addition to the washing buffer at fraction 34 resulted in appearance of a thrombin peak containing only inhibited enzyme. Fig. 3 also includes an experiment in which thrombin inhibited with PPACK was adsorbed on fibrin monomer. Hirudin was then added to the washing buffer, which resulted in elution of the inhibited enzyme. This indicates that modification of the active-centre histidine by PPACK did not prevent thrombin from further interaction with the protein inhibitor, hirudin.

The binding of DAPA to thrombin is accompanied by changes in fluorescence properties of the dansyl moiety, including a 3-fold enhancement in emission intensity

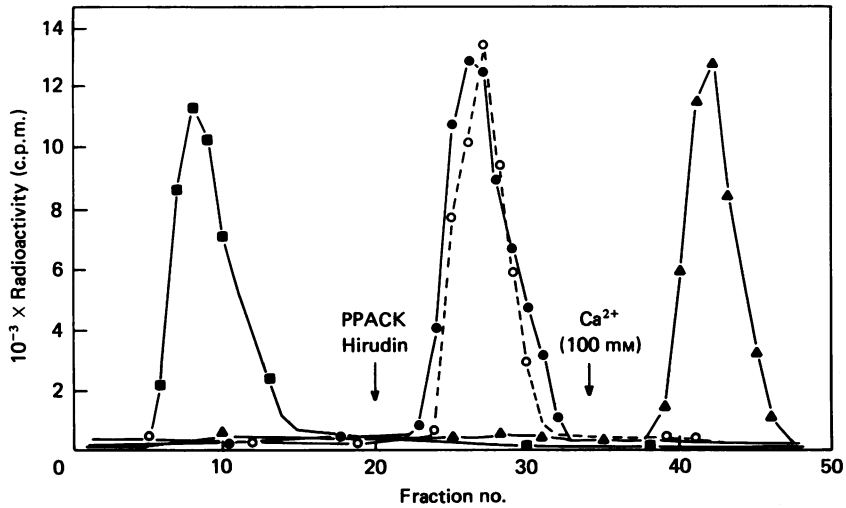

Fig. 3. Effect of hirudin and PPACK on thrombin affinity to fibrin monomer

Fibrin-monomer–Sepharose columns were prepared as in Fig. 1. After sample application, the columns were washed with 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4. At fraction 34, 100 mM-$CaCl_2$ in the same buffer was introduced. ^{125}I-labelled thrombin (15 μg) in 200 μl of washing buffer was treated in the following ways and then chromatographed: thrombin premixed with 50 μl of hirudin (1000 units/ml) (■); thrombin applied, then at fraction 20 hirudin (200 units) added to the elution buffer (●); thrombin premixed with 20 μl of PPACK (86 mM) applied, then at fraction 20 200 units of hirudin added to the washing buffer (○); thrombin applied, then at fraction 20 PPACK (50 μl) added to the washing buffer (▲). At fraction 34 elution with buffer containing 100 mM-$CaCl_2$ was begun. In the radioactivity peaks no clotting activity was detected.

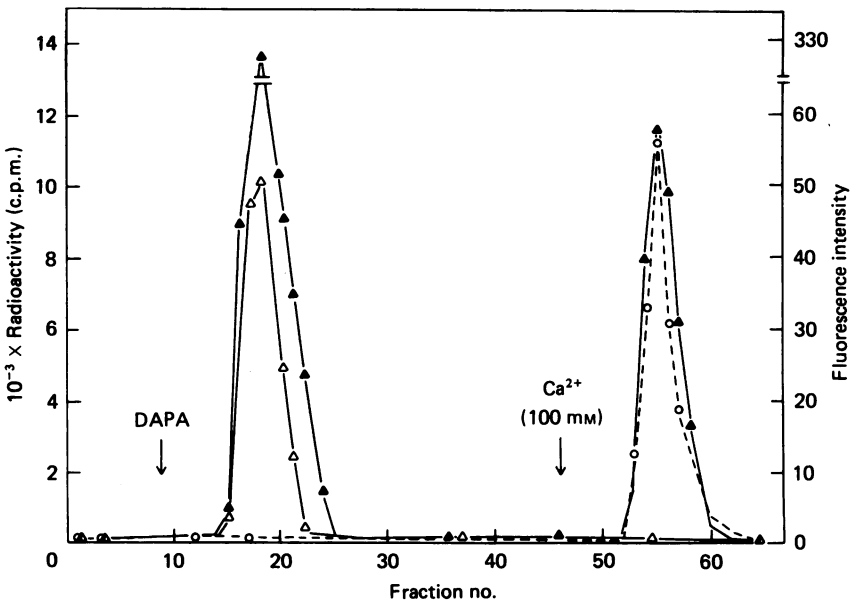

Fig. 4. Inhibition by DAPA of thrombin bound to fibrin-monomer–Sepharose

Fibrin-monomer–Sepharose columns containing 7 ml of gel were prepared as in Fig. 1 and equilibrated with 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4. ^{125}I-labelled thrombin (110 μg in 1 ml) was applied and washing was continued. At fraction 9, 1 ml of DAPA (50 μM) was added to the washing buffer. At fraction 46, elution with 100 mM-$CaCl_2$ was begun. In control experiments, 100 μl of DAPA (50 μM) alone was chromatographed (△). In all fractions fluorescence (▲,△) and radioactivity (○) were measured. Pooled fractions of the radioactivity peak retained approx. 60% of the initial clotting activity.

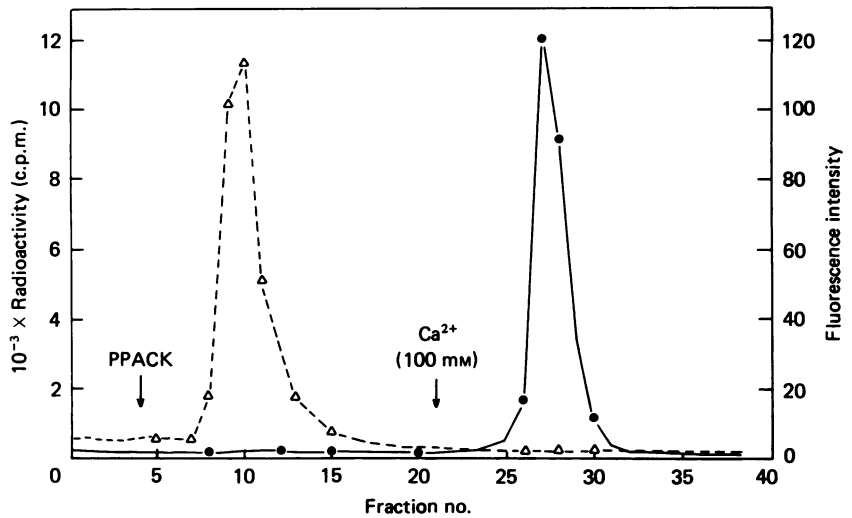

Fig. 5. Displacement of DAPA by PPACK in the active centre of thrombin bound to fibrin monomer

^{125}I-labelled thrombin (1 ml; 80 μg) inhibited with DAPA (50 μM) was applied on a fibrin-monomer–Sepharose column (4 ml of gel). Washing with 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4, was continued until fluorescence returned to the baseline. Then collection of fractions (360 μl, at flow rate of 200 μl/min) was begun. At fraction 4, 50 μl of PPACK (86 mM) was added to the washing buffer. At fraction 21, elution with 100 mM-$CaCl_2$ was begun. In all fractions fluorescence (△) and radioactivity (●) were measured. In the radioactivity peak no clotting activity was detected.

(Nesheim *et al.*, 1979). This property can be used to monitor inhibition, as well as to identify inhibited enzyme in solution. In experiments shown in Fig. 4, ^{125}I-labelled thrombin was applied on fibrin-monomer–Sepharose columns. While the washing was in progress, DAPA was added to the buffer, which resulted in appearance of an unbound fluorescence peak. After fluorescence had returned to the baseline, 100 mM-$CaCl_2$ was added to the buffer, causing elution of another fluorescence peak, this time matched by the radioactivity elution profile. Approx. 40% of the eluted thrombin remained inhibited. In a control chromatography where the sample contained only DAPA, no binding of the inhibitor to fibrin monomer was observed.

Fig. 5 shows the results obtained when a mixture of DAPA and thrombin was applied on a fibrin-monomer–Sepharose column and the column was equilibrated until all unbound fluorescence was eluted. When PPACK was

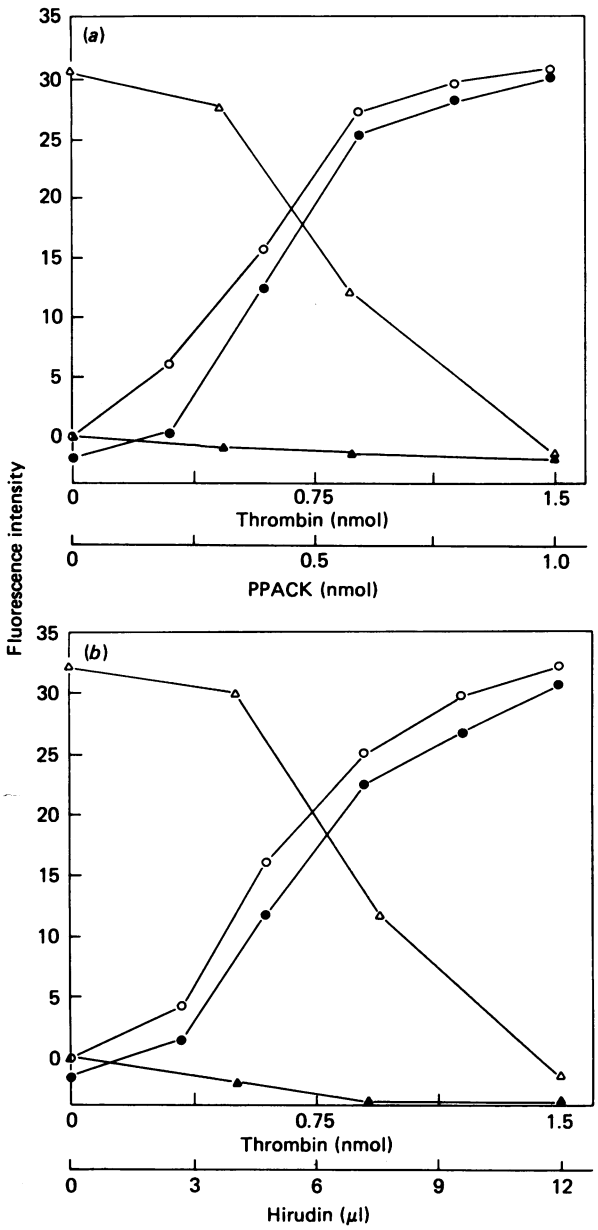

Fig. 6. Displacement of DAPA from the active centre of thrombin in solution by PPACK and hirudin

A cuvette containing 2 ml of DAPA (0.5 μM) was placed in the fluorimeter, and fluorescence intensity was recorded while reaction components were added stepwise in the following order: (1) 4 μl of thrombin (2.8 mg/ml) (●), with enhancement of fluorescence intensity due to thrombin–DAPA complex (note decrease in fluorescence as DAPA is replaced by PPACK or by hirudin); (2) 4 μl of PPACK (86 mM) in (*a*) or hirudin (6000 units/ml) in (*b*) (△); (3) 4 μl of thrombin (○) to titrate the still-available DAPA. Effects of PPACK and hirudin alone on fluorescence were also measured (▲).

added to the washing buffer, a fluorescence peak was eluted without any accompanying radioactivity. The radioactivity was eluted later by $CaCl_2$. There was no fluorescence or clotting activity in this last peak, indicating that PPACK had replaced DAPA in the active centre of thrombin adsorbed on fibrin monomer.

Displacement of DAPA from the active centre of thrombin by PPACK and hirudin in solution

PPACK is an irreversible thrombin inhibitor, whereas DAPA is reversible. Thrombin was added to a cuvette containing DAPA until the increase in fluorescence reached a plateau (Fig. 6*a*); 1 nmol of DAPA was used to titrate 1.53 nmol of thrombin, on the basis of change

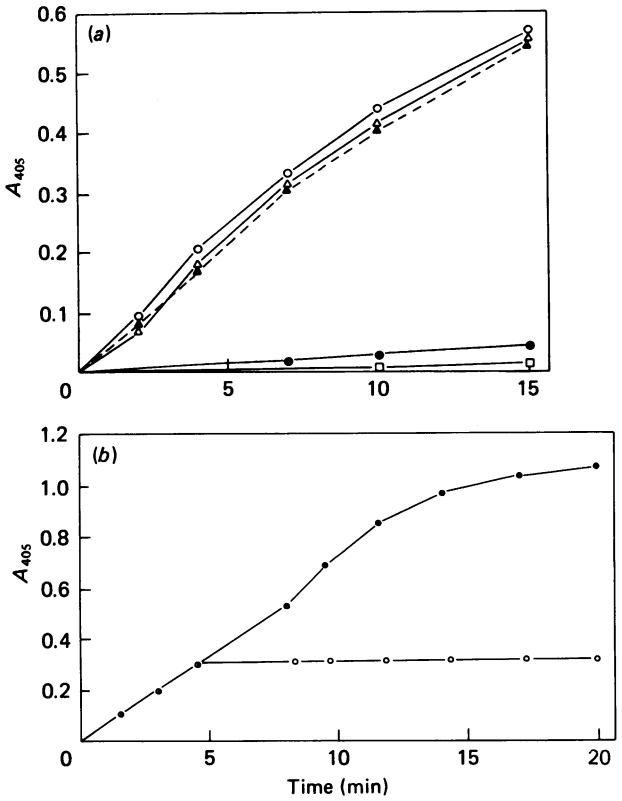

Fig. 7. Hydrolysis of S-2238 by thrombin bound to fibrin-monomer–Sepharose or free in solution

(*a*) To confirm that binding of thrombin to fibrin was complete under the conditions of this experiment, fibrin-monomer–Sepharose (500 μl) in 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4, was equilibrated for 10 min with 500 μl of thrombin (30 units/ml) in the same buffer. After centrifugation (5 min at 1600 *g*), supernatant and gel were separated and mixed with 4 ml of buffer, and 500 μl of 2 mM-S-2238 was added to each tube. At intervals, samples (500 μl) were withdrawn and immediately mixed with 600 μl of PPACK (8.6 μM). A_{405} was measured in samples from the supernatant (●) and gel (○). To compare the rate of hydrolysis of S-2238 by free and fibrin-associated thrombins, 500 μl of plain Sepharose (△) and fibrin-monomer–Sepharose (▲) was pre-equilibrated in separate tubes with 4 ml of 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4, containing 15 units of thrombin. Then 500 μl of S-2238 (2 mM) was added to each tube, and A_{405} was measured. In control experiments with fibrin-monomer–Sepharose, buffer was added in place of thrombin (□). (*b*). For this, 1.0 ml of fibrin-monomer–Sepharose, 1.0 ml of thrombin (30 units/ml) and 7.0 ml of 0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4, were equilibrated for 10 min. Then 1 ml of S-2238 (2 mM) was added. After 4.5 min the reaction mixture was centrifuged (15 s at 1600 *g*) and separated into supernatant only (○) and gel plus supernatant (●). Samples were withdrawn at various times and A_{405} was measured.

in fluorescence intensity. Then PPACK was added to the system, resulting in return of the fluorescence intensity to the initial value. Exactly 1 nmol of PPACK displaced 1 nmol of DAPA in the active centre of thrombin. To determine whether the released DAPA was still functional, thrombin was added a second time to titrate DAPA. An increase in fluorescence intensity, parallel to the initial one, confirmed the previous observation that interaction of DAPA with thrombin does not alter the structure of this inhibitor (Kettner & Shaw, 1979). The same results were obtained when hirudin was used instead of PPACK (Fig. 6*b*).

Hydrolysis of S-2238 by thrombin in solution or bound to fibrin monomer

Experiments described in Fig. 7 were based on the observation that, under the conditions chosen (0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4), thrombin binding to fibrin-monomer–Sepharose was complete, and no free thrombin remained in the fluid phase. To confirm this, fibrin-monomer–Sepharose was first equilibrated with thrombin; after centrifugation the gel and supernatant were separated, diluted, and made to react with S-2238. Essentially no hydrolysis was detected in the tube containing supernatant (Fig. 7*a*). S-2238 hydrolysis rates by bound and by free thrombin were then determined. To achieve this, measured amounts of fibrin-monomer–Sepharose or plain Sepharose were equilibrated with the same amount of thrombin, and then synthetic substrate was added to each tube. Hydrolysis rates were measured in samples withdrawn at various times, and no difference was observed between the two test systems (Fig. 7*a*). To exclude the possibility that S-2238 may dissociate thrombin from fibrin monomer, the experiment shown in Fig. 7(*b*) was performed. To fibrin-monomer–Sepharose pre-equilibrated with thrombin, S-2238 was added, and the amount of hydrolysed substrate was measured. After 4.5 min the test tube was rapidly centrifuged and the contents were separated into two fractions, one containing gel and supernatant and the other with supernatant only. Measurements of hydrolysis in both fractions showed that the reaction proceeded only in the tube containing fibrin-monomer–Sepharose. In the tube with supernatant only, the amount of hydrolysed substrate remained constant at the value reached before centrifugation.

DISCUSSION

In this study we have further investigated thrombin binding to fibrin, particularly with respect to the role that the enzyme active centre may play in this interaction. A variety of thrombin-specific inhibitors, together with measurement of enzyme activity for a specific synthetic substrate, have been used to define activity of thrombin complexed with fibrin monomer.

Previous studies demonstrated that in a non-flowing fibrinogen-clotting system thrombin, in which the active centre was inhibited by PMSF and di-isopropyl fluorophosphate, was still able to bind to fibrin (Fenton *et al.*, 1981; Wilner *et al.*, 1981). Our results confirm this observation in a test system using fibrin-monomer–Sepharose columns with ligand added in the fluid phase (Fig. 1). These studies also show that there is no apparent difference in binding properties of active and PMSF-inhibited thrombins to fibrin monomer. Furthermore, in a column containing fibrinogen–Sepharose, the elution pattern of PMSF-blocked thrombin was identical with that of active thrombin, suggesting that prior cleavage of fibrinopeptide does not affect the equilibrium binding, at least in test systems where fibrinogen and fibrin-monomer molecules are immobilized on agarose beads.

PPACK (M_r 576) is a water-soluble, irreversible, highly specific inhibitor of thrombin, which blocks the active-centre histidine (Kettner & Shaw, 1979). As demonstrated in Fig. 3, inhibition with this reagent does not affect thrombin affinity for fibrin. In this respect, PPACK- and PMSF-treated thrombins are indistinguishable, although PMSF blocks the active-centre serine. Another low-M_r inhibitor, DAPA, also does not interfere with thrombin affinity to fibrin. This inhibitor, even though very specific, is reversible and does not modify the active-centre amino acids. Presumably its arginine interacts electrostatically with carboxy groups in the binding pocket (Nesheim *et al.*, 1979). Hence these three active-site inhibitors, with different mechanisms of inhibition, all inhibit both the fibrinogen-clotting activity and esterase activity of thrombin, but have no effect on the binding of thrombin to fibrinogen or fibrin monomer.

The only low-M_r inhibitor tested that interferes with thrombin binding to fibrin monomer is pyridoxal 5′-phosphate. During covalent modification of human thrombin with this reagent, two lysine residues may be involved, but the specific ones have not yet been determined. However, the presence of heparin in the reaction mixture protects the second site, and therefore only the first lysine undergoes modification (Griffith, 1979). Heparin-protected thrombin, with a single lysine modified, was used in this investigation. This modified enzyme, although unable to clot fibrinogen, retains its esterolytic activity towards synthetic substrates and its ability to interact with antithrombin III (Griffith, 1979). The results presented here show that it is unable to bind to fibrin (Fig. 2). After the modification procedure, phosphopyridoxylated thrombin contained an appreciable amount of active enzyme (approx. 15%), and chromatography on fibrin-monomer–Sepharose clearly separated the inactive from the active fraction. Hence, this affinity chromatography can be used as a final step in the modification procedure to obtain a population of totally inactive molecules. In practice, decreasing the ionic strength of the washing buffers (0.01 M-Tris/HCl/0.02 M-NaCl, pH 7.4) will increase the efficiency of separation to the degree that the first unbound peak will contain exclusively pyridoxal 5′-phosphate-inhibited thrombin.

Hirudin has been used as a high-M_r inhibitor in this study. As demonstrated in Fig. 3, it is clear that preformed thrombin–hirudin complexes do not interact with fibrin monomer, which is consistent with previous observations (Fenton *et al.*, 1981; Wilner *et al.*, 1981). Similar results were also obtained with thrombin–antithrombin III complexes (Kaminski & McDonagh, 1982).

The question of the extent to which the active centre of thrombin associated with fibrin is still accessible to its substrates and inhibitors has also been examined. We have observed that the water-soluble low-M_r inhibitors, PPACK and DAPA, can readily modify the active centre of the fibrin-associated enzyme and that the active centre

is exposed to the degree that displacement of DAPA by PPACK is possible without disturbing the binding (Figs. 5 and 6). Furthermore, the experiments shown in Fig. 7 indicate that thrombin bound to fibrin-monomer–Sepharose hydrolyses a synthetic tripeptide substrate at the same rate as the free enzyme. These data clearly indicate that the active centre of thrombin bound to fibrin monomer is fully exposed and freely accessible; it can react with a variety of inhibitors ranging in M_r from 578 (PPACK) to 56000 (antithrombin III). This suggests the possibility that interaction with fibrin monomer may have a regulatory role in the activation of other coagulation factors by thrombin and its inactivation by antithrombin III.

This work was supported in part by N.I.H. Grant HL 33014, from the National Institutes of Health.

REFERENCES

Berliner, L. J. & Sugawara, Y. (1985) Biochemistry **24**, 7005–7009
Blombäck, B., Hessel, B., Hogg, D. & Therkildsen, L. (1978) Nature (London) **257**, 501–505
Carney, D. H., Glenn, K. C., Cunningham, D. D., Das, M., Fox, C. F. & Fenton, J. W., II (1979) J. Biol. Chem. **254**, 6244–6247
Carr, M., Jr., Kaminski, M., McDonagh, J. & Gabriel, D. A. (1985) Thromb. Haemostasis **54**, 159
Fenton, J. W. II, Landis, B. H., Walz, D. A., Bing, D. H., Feinman, R. D., Zabinski, M. P., Sonder, S. A., Berliner, L. J. & Finalson, J. S. (1979) in The Biochemistry and Physiology of the Human Plasma Proteins (Bing, D. H., ed.), pp. 151–183, Pergamon Press, New York
Fenton, J. W., II, Zabinski, M. P., Hsieh, K. & Wilner, G. D. (1981) Thromb. Haemostasis **46**, 177
Francis, C. W., Marder, V. J., Barlow, G. H. & Martin, E. (1981) Thromb. Haemostasis **46**, 177
Griffith, M. J. (1979) J. Biol. Chem. **254**, 3401–3406
Heene, D. L. & Matthias, F. R. (1973) Thromb. Res. **2**, 137–154
Kaminski, M. & McDonagh, J. (1982) Circulation **66**, 11–294
Kaminski, M. & McDonagh, J. (1983) J. Biol. Chem. **258**, 10530–10535
Kaminski, M. & McDonagh, J. (1985) Thromb. Haemostasis **54**, 294
Kettner, C. A. & Shaw, E. N. (1979) Thromb. Res. **14**, 969–973
Liu, C. Y., Nossel, H. L. & Kaplan, K. L. (1979) J. Biol. Chem. **254**, 10421–10425
Liu, C. Y., Nossel, H. L. & Kaplan, K. L. (1981) Thromb. Haemostasis **46**, 177
Liu, C. Y., Handley, D. L. & Chien, S. (1985) Anal. Biochem. **147**, 49–56
Marsh, H. C., Jr., Meinwald, Y. C., Lee, S. & Sheraga, H. A. (1982) Biochemistry **21**, 6167–6171
Nagy, J., Meinwald, Y. C. & Sheraga, H. A. (1982) Biochemistry **21**, 1794–1806
Nesheim, M. E., Prendergast, F. J. & Mann, K. G. (1979) Biochemistry **18**, 996–1003
Wilner, G. W., Danitz, M. P., Mudd, S., Hsieh, K. H. & Fenton, J. W., II (1981) J. Lab. Clin. Med. **97**, 403–411

Received 16 July 1986/7 October 1986; accepted 25 November 1986